Synapse formation: on the role of extrasomal compartments

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on the role of extrasomal compartments

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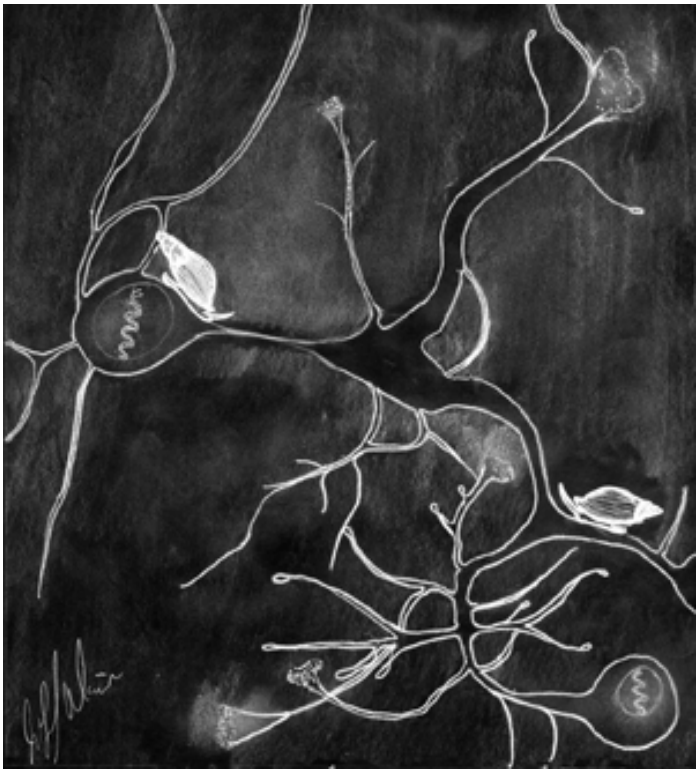

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2005

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General Introduction

1. Introduction

Neuronal ensembles orchestrated during development control all animal functions ranging from overt behavior to learning and memory. Thus, a comprehensive knowledge of various cellular and molecular mechanisms by which networks of neurons are assembled during development is pivotal for our understanding of how the brain controls various animal functions after birth. This information is also essential to cure various nervous system dysfunctions that are met in clinical practices. To achieve its highly specialized connectivity pattern, an immature neuron undergoes several developmental changes before it reaches its designated function in the nervous system. Following proliferation and migration a neuron must extend its axonal or dendritic growth cones towards targets that are generally located at some distance. A highly sophisticated pathfinding program enables the growth cone to navigate through the extracellular milieu to reach the vicinity of its target cells. Upon contact with target cells, synapses develop, mature and function, thereby finally generating highly specialized connectivity patterns which are essential for all brain functions. This thesis deals primarily with the elucidation of cellular mechanisms underlying synapse formation, however, all developmental steps are highly intertwined and a collective, albeit brief description may be deemed central to our understanding of how a neuron completes its journey from neurite outgrowth to synaptogenesis. A detailed description of all developmental events is beyond the scope of this thesis and the reader is directed towards some excellent reviews in this field (Markus et al., 2002; McAllister, 2002; Boyd and Gordon, 2003; Marzella and Gillespie, 2002; Huang and Reichardt, 2001; Yamamoto et al., 2002). This chapter of the thesis will thus primarily highlight our current understanding of both cellular and molecular steps involved only in axonal pathfinding, target cell selection, synapse formation and synaptic refinement.

1.1. Neuronal Development

1.1.1. From growth cones to synapses.

Axonal pathfinding is primarily regulated by a variety of intrinsic cellular programs and various extrinsic factors that are present within its environment. Axonal and dendritic cytoskeleton provides the backbone upon which neuronal polarity is built and this structural framework subsequently serves to transport housekeeping proteins to and from the cell body. Among the proteins that constitute the neuronal cytoskeleton are the microtubules, microfilaments and neurofilaments. The dynamic assembly of these three primary components gives rise to axonal and dendritic polarity during development and maintains their structural integrity in the adult brain. Although the cytoskeletal organizations of both axonal and dendritic proteins somewhat differ, their elongation during development does, nevertheless, hinge upon growth cones located at the very tip of these processes. Fine, finger like projections termed the filopodia, extend from the main body of the growth cone (lamellipodia) and sample the milieu as a sophisticated network of tentacles or antennae. As the growth cone extends, the fluidic microtubular machinery gets firmly assembled into bundles of organized tracks, transforming a highly motile structure into a firm neurite.

Distinct sets of membrane proteins, such as receptors, ion channels, transporters and adhesion molecules are present within the growth cones prior to contact with its target cell. The maintenance of neuronal polarity relies upon scaffolding proteins, which together with the cytoskeletal matrix, form a barrier within the membrane to prevent protein diffusion (Kobayashi et al., 1992; Winckler et al., 1999; Nakada et al., 2003; Borgdorff and Choquet, 2002; Serge et al., 2002), while stabilizing and targeting various protein complexes at the membrane (Harris and Lim, 2001; Jacob and Naim, 2001; Keller et al., 2001; Kreitzer et al., 2003).

The neuronal polarity is also regulated by external factors such as neurotrophins. For instance, dendritic morphology of vertebrate cortical neurons is influenced by neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) (McAllister et al., 1995; McAllister et al., 1997). BDNF, a member of the neurotrophin family of growth factors, can also induce axon outgrowth in the developing CNS (Lom and Cohen-Cory, 1999; Cohen-Cory and Fraser, 1995) while altering the polarity of dendtritic spines in the adult mammalian CNS (McAllister et al., 1995; McAllister et al., 1997; Shimada et al., 1998; Lom and Cohen-Cory, 1999; Horch et al., 1999; Yacoubian and Lo, 2000). BDNF released locally by dendrites and cell bodies of cortical neurons in a ferret brain slice, increases dendritic branching of nearby neurons in a gradient-dependent manner (Horch and Katz, 2002). These studies serve to suggest that extrinsic trophic factors not only affect neuronal polarity during development but also in the adult brain. Both cellular and molecular mechanisms by which trophic factors affect other developmental events will be discussed later in detail.

In addition to providing a substrate for neurite extension, the growth cones also house various components of the preassembled synaptic machinery. A recent study on transfected primary cultures of postnatal rat visual cortical neurons has provided interesting evidence in support of the idea that filopodia may also serve important roles during synapse formation. In contrast with the conventional view that the filopodia are devoid of membranous organelles (Tennyson, 1970; Gordon-Weeks, 2004), this study demonstrates them to contain transmitter vesicles (Sabo and McAllister, 2003). These filopodial vesicles exhibit functional properties and molecular profiles that are characteristic of synaptic vesicles at mature synapses. Moreover, these vesicles move bidirectionally – thus adding additional dynamic to the neuronal polarity. For example, during axonal pathfinding and target selection, these vesicles can be directed (growth factors, membrane proteins etc.) towards filopodial surface for a rapid assembly of synaptic proteins during synapse formation. In support of this, proteins of the active zones (AZs)-sites for presynaptic transmitter vesicle exocytosis have been demonstrated to be harbored in the Piccolo-Bassoon transport vesicles (PTV). The content of an individual vesicle is on average half of that of an individual presynaptic bouton, and it is therefore suggested that presynaptic AZ sites might be formed by the fusion of a few PTVs into the presynaptic membrane (Shapira et al., 2003).

In addition to the above-described intrinsic and extrinsic diffusible factors, various membrane bound proteins comprise a complex of extracellular matrix (ECM) and cell adhesion molecules (CAMs), which assist growth cones in their navigational tasks.

ECM molecules

ECM is comprised of a variety of molecules such as laminin, fibronectin, and collagen. For the sake of simplicity and focus, the function of only laminin and its involvement in neuronal outgrowth will be described for an example. The reader is directed to several excellent reviews on the role of other ECM molecules (Sobeih and Corfas, 2002; Corey and Feldman, 2003).

Laminin, a glycoprotein of the ECM, not only promotes growth and differentiation of neurons but also serves to guide axons towards their targets. Both *in vivo* and *in vitro*, laminin regulates the direction and the rate of neurite outgrowth through its interactions with growth cones. Laminin is synthesized and secreted into the ECM and interacts with receptors on cell surfaces to affect migration and neurite outgrowth. The laminin protein is comprised of various different domains and each is associated with distinct functions such as cell attachment, neurite outgrowth and interactions with other glycoproteins and proteoglycans. Various receptors and binding proteins on cell surfaces can interact with laminin (Mecham, 1991), exhibiting varying degrees of binding affinities. In addition, laminin can bind to several receptors and some receptors in the ECM can bind to several different proteins on the cell surface, suggesting that no single laminin-receptor interaction is solely responsible for mediating cellular responses, rather there may exist a hierarchical organization for various cellular response mechanisms (Begovac et al., 1991). Among various laminin receptors are (1) β-integrins, (2) proteins that bind specific sequences in one of the polypeptide chains, and (3) carbohydrate-binding moieties such as lectins and galactosyltransferase. Besides laminin, tenascin and fibronectin are also members of the ECM proteins that regulate neurite outgrowth (Bixby and Harris, 1991). In addition to their direct role in directing the migration of neuronal cells, the ECM might be involved in the binding of neurotrophins, presenting them to the neurons en-route to their final destination (Barnett et al., 2002; Rider, 2003). While various ECM proteins pave the way for pioneer axons, the growth of subsequent neurons is facilitated through cell-cell interactions either between the axons/dendrites themselves, or via other cell types such as the glia.

Cell adhesion molecules

The interactions between various cell adhesion molecules (CAMs) located at the surface of growth cones, and their corresponding receptors on other neuronal or non-neuronal cells and vice-a-versa, are also critical for proper projections of all neurons in the nervous system (Dodd and Jessell, 1988; Goodman and Shatz, 1993). The neuronal receptors are the β1-integrins, which recognize ECM molecules, and N-CAM, N-cadherin, and L1 glycoprotein (Bixby and Harris, 1991; Walsh and Doherty, 1997), which promote growth over non-neuronal cells. At different developmental stages, CAMs fulfill different roles. For instance, during early developmental stages N-CAM promotes cell migration, axonal growth and synaptic plasticity, whereas at later stages, it serves to stabilize synaptic connections (Mayford et al., 1992; Cremer et al., 1997). Reason for this switch in function is the role of VASE exon, which adds 10 amino acids into the N-CAM molecule during CNS development. This increased activity of the VASE exon correlates with a loss of N-CAM-dependent neurite outgrowth (Walsh et al., 1992; Doherty et al., 1992). During development the increased use of VASE, therefore, seems to be involved in switching the function of N-CAM from growth promotion to synaptic stability. Other

CAMs such as the ones belonging to the cadherin family, have been proposed to be important for developmental processes such as regionalization, brain nucleus formation, neurite outgrowth, target recognition and synaptogenesis (Hirano et al., 2003). While various ECM molecules and CAMs are instrumental in promoting growth, a variety of other factors such as semaphorins, neurotransmitters, Eph receptors and their ligands, designated as guidance molecules, serve simultaneously as growth permissive and repulsive cues to escort neurons towards their target sites (Pasterkamp and Verhaagen, 2001; Spencer et al., 2000b; van Kesteren and Spencer, 2003; Zisch and Pasquale, 1997).

1.1.2. Target cell selection

Upon approach to its appropriate target cell, neurite extension is terminated and synapses begin to develop (Kapfhammer and Raper, 1987a,b; Cooper and Smith, 1992; Fawcett, 1993). This suggests that contact between appropriate cells triggers a molecular synaptic program that on one hand enhances synapse formation and on the other hand terminates neurite outgrowth. Because, growth cones are able to release transmitters prior to their contact with specific targets (Hume et al., 1983; Young and Poo, 1983), it is hypothesized that such molecules may diffuse across the membrane to affect the growth of its potential partner. For instance, local release of glutamate stabilizes transient interactions between axonal and dendritic filopodia, thus facilitating synapse formation (Jontes and Smith, 2000). In support of this view are further studies that show that neurotransmitters can indeed suppress the motility of axonal and dendritic filopodia (Chang and De Camilli, 2001; Lin and Constantine-Paton, 1998; Wong and Wong, 2001), thereby slowing down the growth of axons. In addition to terminating the neurite extension associated with synapse formation, a variety of neurotransmitters and their receptors have also been shown to act as growth repulsive molecules thus preventing axons from entering the wrong territory. This might ensure to prevent contact between inappropriate partners thus maintaining the specificity of synaptic connections.

Intracellularly, the stabilization of newly contacted processes requires re-arrangement of the actin and microfilament cytoskeleton of the growth cone. This rapid and transient redistribution can be induced by the activation of the neurotrophin and Eph (erythropoietin-producing hepatocellular) receptor tyrosine kinases (Gallo et al., 1997; Meima et al., 1997). The Eph receptor tyrosine kinase (RTK) subfamily and their ligands direct the formation of the appropriate connections (Friedman and O'Leary, 1996; Tessier-Lavigne, 1995). A protein that mediates the redistribution of the cytoskeleton is L1, a cell adhesion molecule that binds a number of proteins including ECM molecules (Burden-Gulley et al., 1997). Other proteins such as HB-GAM (heparin-binding growth-associated molecule), a component of the extracellular matrix, and cadherins (Fannon and Colman, 1996) have also been shown to play crucial roles in target cell selection. This notion is consistent with the expression patterns of various splice variants of cadherins that are expressed in the developing CNS (Redies and Takeichi, 1996) and are localized to specific synapses (Redies and Takeichi, 1996; Uchida et al., 1996). Classic cadherins bind preferentially the same cadherin subtype, providing an "adhesive code" for various aspects of neuronal morphogenesis.

The above cited examples serve to highlight some aspects of target cell selection, however, for a detailed description of various molecular guidance cues and their

downstream mechanisms, the reader is directed towards some excellent recent reviews (Guan and Rao, 2003; and Araújo and Tear, 2003).

1.1.3. Synapse Formation

Prior to physical contact between partner cells, several components of the synaptic machinery are believed to be already 'pre-assembled' in the outgrowing neurite (Ahmari et al., 2000), or contained in Piccolo-Bassoon transport vesicles (PTVs) (Shapira et al., 2003). However, contacts between specific pre-and postsynaptic partners have been shown to bring about dramatic morphological, cellular, and molecular changes (Haydon and Drapeau, 1995; Fitzsimonds and Poo, 1998; Ferreira and Paganoni, 2002) that are the hallmark of specialized synaptic sites. Notwithstanding the fact that several molecules and the underlying mechanisms involved in axon outgrowth and target cell selection also function during synapse formation, many other molecules are specifically dedicated for synapse formation. Various molecular components of the synaptic assembly and the underlying mechanisms are well defined at the neuromuscular junction (NMJ) which has contributed significantly to our understanding of the fundamental principles that regulate synapse formation. However, as compared with the NMJ, less understood are the mechanisms that govern synapse formation in the central nervous system (CNS), though some generalities can be drawn.

1.1.3a. Synapse Formation at the Neuromuscular Junction.

The NMJ has long been considered as a useful model system for studies on synapse formation. For instance, already in 1968 James and Tresman had demonstrated that specific synapse between motor neurons and their muscle partners can be reconstituted in cell culture (James and Tresman, 1968). Since then, the NMJ has revealed numerous principles underlying synapse formation and have identified both intrinsic and extrinsic cellular and molecular molecules that generate synapse specificity in this model. Although a number of molecules and their underlying mechanisms have been identified and well described (Sanes and Lichtman, 1999; Huh and Fuhrer, 2002), only the key players will be discussed here.

Postsynaptic differentiation.

The most prominent changes during earlier synaptic differentiation are clearly discernable on the postsynaptic membrane. These include receptor clustering, development of postsynaptic cytoskeleton, and the elaboration of synapse-specific extracellular matrix soon after the arrival of motor neuron. However, some components of this postsynaptic machinery gather at the pre-defined synaptic sites even prior to the arrival of motor neurons, suggesting that muscles can regulate some aspects of its synaptic development independent of motor neuron-derived signals (Yang et al., 2000, 2001; Lin et al., 2001; Arber et al., 2002). However, the necessity of this prepatterning for synapse formation is not clear, since *in vitro* synapse formation can proceed in the absence of muscle-derived positioning of the postsynaptic apparatus (Sanes and Lichtman, 1999). The next event in synapse formation is the release of agrin from the arriving motor neuron. Agrin, a proteoglycan, induces postsynaptic specializations on the muscle fiber (McMahan, 1990; Sanes and Lichtman, 2001). Once deposited in the basal lamina, agrin induces further clustering and stabilization of prepatterned ACh receptors at

the innervation site. The receptor for agrin is MuSK, which is a transmembrane receptor tyrosine kinase. MuSK plays a crucial role in agrin-induced ACh receptor clustering, because in MuSK knock-out mice no signs of postsynaptic differentiation have been detected, even though the ACh receptor expression appears normal (DeChiara et al., 1996). The effector protein of MuSK is rapsyn, which is a membrane associated cytoplasmic protein. Rapsyn is tightly associated with ACh receptors and appears at the NMJ as soon as they cluster and co-localizes with them at the adult NMJ (Noakes et al., 1993). Muscles from rapsyn knock-out mice do not exhibit ACh receptor clustering. Moreover, myofibers from knock-out mice treated *in vitro* with various AChR clustering agents also fail to induce receptor clustering (Gautam et al., 1995), strongly suggesting that rapsyn is a necessary participant in the agrin-MuSK-rapsyn-ACh receptor pathway for ACh receptor clustering. Specifically, rapsyn and AChR proteins are co-transported and targeted to the innervated surface of the *Torpedo* electrocyte (Marchand et al., 2000). Co-transfection of rapsyn and ACh receptor subunits in COS-7 cells suggests that rapsyn not only associates with ACh receptors on the cell surface, but also escorts ACh receptors to the cell surface (Marchand et al., 2002).

Selective transcription of ACh receptor genes by synapse-associated myonuclei also contributes to subsequent and sustained synaptic accumulation of ACh receptors. ARIA (AChR-inducing activity) is an isoform of the secreted growth factor neuregulin-1, a neuronal factor that stimulates AChR synthesis by myotubes (Falls et al., 1993; Fischbach and Rosen, 1997). Similarities exist between the agrin and the neuregulin pathway in that neuregulin is also synthesized and expressed by the motoneuron and secreted into the synaptic cleft. In addition, neuregulin receptors, like MuSK, are transmembrane tyrosine kinases (ErbB kinases) that are concentrated at the postsynaptic NMJ membrane (Trinidad et al., 2000; Moscoso et al., 1995; Rimer et al., 1998; Zhu et al., 1995). These studies have led to the idea that agrin and neuregulin act in parallel as nerve-derived signals. Agrin triggers the clustering of AChRs and neuregulin activates localized AChR transcription at the myofibers. On one hand, agrin-MuSK signaling is as simple as any other growth factor-receptor interactions with the exception that agrin does not directly bind to MuSK. MuSK expressed on non-muscle cells does not become phosphorylated upon agrin binding, whereas recombinant and endogenous MuSK expressed on myotubes do (Glass et al., 1996). Therefore, MuSK is thought to form a complex with another molecule that is selectively expressed in muscle fibers and is essential for agrin binding (Glass et al., 1996). This hypothetical molecule is called myotube-associated specificity component (MASC). MASC and MuSK together in a complex form a high-affinity receptor for agrin. Binding of agrin to this MuSK/MASC complex should trigger phosphorylation of MuSK, the typical response of tyrosine kinases upon binding of their ligand, followed by subsequent intracellular cascade of events that lead eventually to transcription of the appropriate proteins required for the formation of mature synapses (Apel and Merlie, 1995). Similarly, binding of neurotrophins to their Trk tyrosine kinase receptors also modulates the size and shape of the NMJ postsynaptic membrane. This role of neurotrophins is possibly related to the one they exert in CNS synapse formation (Gonzalez et al., 1999; Belluardo et al., 2001; Wells et al., 1999).

Taken together, the above studies have demonstrated that various presynaptic cell-derived molecules can influence postsynaptic differentiation to make the future synaptic transmission compatible with the physiological needs.

Presynaptic differentiation.
As mentioned earlier, some components of the presynaptic machinery are pre-assembled prior to contact with synaptic partners. However, the functional organization of many of the proteins and the formation of synaptic specializations require postsynaptic signaling. These specializations include clustering of synaptic vesicles, targeting of Ca^{2+}-channels, and assembly of protein complexes responsible for excitation-secretion coupling (Herlitze et al., 2003; Spafford and Zamponi, 2003). Agrin and MuSK knock-out mice experiments have also demonstrated that in the absence of the postsynaptic machinery components, the presynaptic apparatus fails to develop suggesting its involvement in retrograde signaling. The motoneurons in these mutant mice do not settle down to differentiate but remain highly dynamic and motile (Gautam et al., 1996; DeChiara et al., 1996). Further, transplantation of MuSK knock-out muscle fibers into wild-type animals results in continuous remodeling of the muscle-contacting motoneuron which remain undifferentiated for several months (Nguyen et al., 2000). Although the identity of postsynaptic signals that are necessary to induce presynaptic differentiation have not been clearly identified (Sanes and Lichtman, 1999), laminins appear necessary for certain aspects of presynaptic development. Laminins deposited in the synaptic basal lamina seem to be required for presynaptic differentiation as well as for synaptic alignment. Specifically, presynaptic differentiation is compromised in laminin β2 knock-out mice (Noakes et al., 1995) and laminin α4 knock-out mice show normal synaptic differentiation to a certain extent, however, the pre-and postsynaptic specializations are frequently misaligned (Patton et al., 2001). Since laminin α4 can be biochemically linked to presynaptic calcium channels, this protein is thought to be involved in *trans*-synaptic alignment (Sunderland et al., 2000). In summary, synapse formation at the NMJ probably follows the following order of events: 1) agrin is released by the approaching presynaptic motoneuron and 2) induces MuSK activation and phosphorylation on the postsynaptic muscle fibre (Figure 1). Subsequenlty, the muscle fiber deposits laminins and possibly other signaling molecules into the synaptic basal lamina, which in turn induces presynaptic specialization.

1.1.3b. Synapse Formation in the Central Nervous System
As compared to the NMJ, both the molecular machinery and the mechanisms underlying synapse formation at central synapses are not well understood. For instance, various agrin isoforms have been found in the CNS (Hoch et al., 1993; Stone and Nikolics, 1995; Cohen et al., 1997), and during active synaptogenesis agrin mRNA expression is upregulated in response to neuronal activity. Therefore, it has been suggested that agrin also regulates synapse formation in the brain (Cohen et al., 1997). This is supported by agrin suppression experiments in cultured hippocampal neurons that show severely compromised synapse formation (Ferreira, 1999; Böse et al., 2000). However, lack of agrin does not completely block synapse formation and in primary hippocampal and cortical neurons cultured from agrin-deficient mice synapses develop normally (Li et al., 1999; Serpinskaya et al., 1999), suggesting that agrin plays more of a modulatory rather

than a regulatory role in synaptogenesis. Conversely, MuSK has not been detected in the CNS (Ganju et al., 1995; Besser et al., 1996) though its closest relatives, the orphan receptor tyrosine kinases ROR1 and ROR2, have been identified as neuronal proteins (Masiakowski and Carroll, 1992). Despite the presence of agrin and MuSK relatives in the CNS, their precise involvement in synapse formation has yet to be determined. For example, cultured mouse neurons still develop normal synapses in the absence of various active agrin isoforms (Serpinskaya et al., 1999; Li et al., 1999). In addition, in superior cervical ganglion (SCG,) neuronal rapsyn proteins do not co-express with the ACh receptor clusters. Most convincingly, in SCGs of rapsyn-deficient mutant mice both synaptic and nonsynaptic ACh receptor clusters formed normally (Feng et al., 1998). In the CNS other proteins have been identified that do not share sequence homology with rapsyn, but that do share some functional properties with rapsyn.

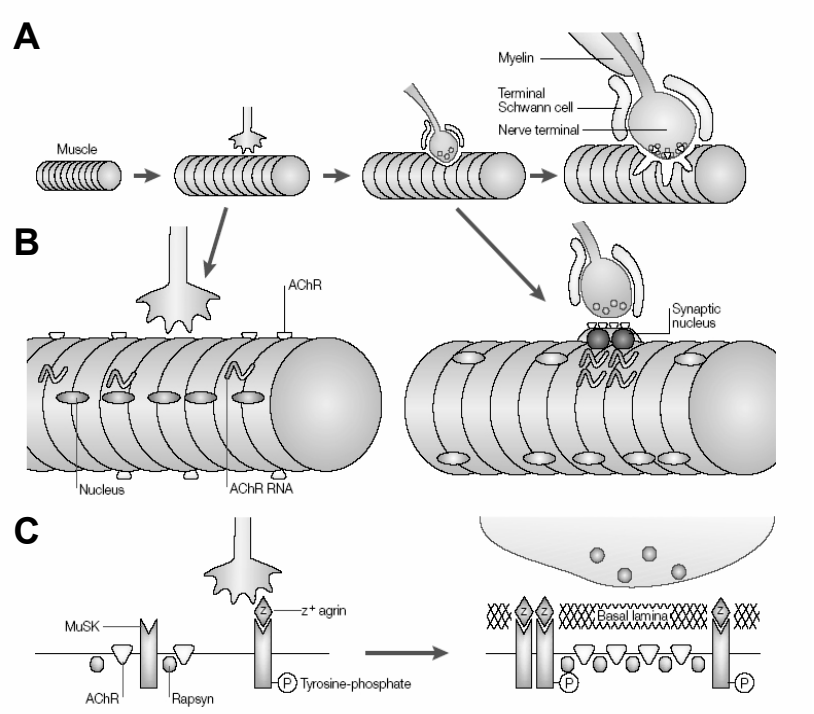

Figure 1: Synapse formation at the Neuromuscular Junction.
An overview of various steps involved in synapse formation at the neuromuscular junction. (**A**) The approaching motor axon growth cone transforms into a specialized motor nerve terminal upon contact with the muscle fiber. (**B**) AChRs are re-distributed to the contact site, as well as locally synthesized at the motor axon-muscle innervation site. (**C**) Agrin-MuSK-rapsyn interact to induce AChR clustering. Agrin, released from the nerve terminal, activates MuSK and clusters AChRs through rapsyn. (Figure is taken from Sanes and Lichtman, 2001).

Gephyrin, that has been shown to be essential for the formation of glycinergic membrane specializations (Kirsch et al., 1993), and PSD-95 are two examples of proteins that associate with the inhibitory glycine and N-methyl-D-aspartate (NMDA) receptors respectively, suggesting a rapsyn-like function for these proteins.

The above studies thus serve to suggest that molecules that are critical for synapse formation at the NMJ are either not expressed in the CNS or they fail to contribute significantly to synapse formation. However, genetically and functionally related proteins have been identified in the CNS that could exert similar effects on the development of pre- and postsynaptic specializatons, though the precise mechanisms remain poorly defined.

Postsynaptic differentiation

Growing axons are capable of neurotransmitter release prior to contact with postsynaptic targets (Sun and Poo. 1987; Kraszewski et al., 1995). At the time of synapse formation, presynaptic electrical activity induces release of glutamate which in turn promotes dendritic filopodial motility (Dailey and Smith, 1996; Lendvai et al., 2000; Wong et al., 2000). Axon filopodia motility is also enhanced by glutamate or electrical stimulation, which is mediated by kainate receptors (Tashiro et al., 2003) or AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)-type glutamate receptors (De Paola et al., 2003). Coordination of the enhanced dendritic and axonal filopodia motility by localized glutamate release increases the chances of axo-dendritic contacts thus facilitating the synapse formation. In contrast, several studies have shown a decrease in filopodial motility by glutamate. For instance, activation of cultured hippocampal neurons through AMPA or kainate receptors blocks dendritic spine movement (Fischer et al., 2000) and axonal growth cone filopodia (Chang and De Camilli, 2001). In mature hippocampal culture slices, in contrast with young cultures, mossy fiber filopodia motility is reduced by kainate receptor activation (Tashiro et al., 2003). Since neurotransmitter release can either stabilize or increase filopodia motility at different stages of development, it is plausible that during early development released neurotransmitters may increase filopodial motility to stimulate growth and the rate of advance of outgrowing neurons, whereas once neurons come in contact with their targets, the secreted neurotransmitters are more likely to stabilize filopodia for synapse formation that follows. In contrast, a recent study suggests another role for neurotransmitters in neuronal development. Munc-18 knock-out mice that lack regulated transmitter release exhibit normal neuronal development and synaptic connectivity. However, although transmitter release does not seem to be important for the formation of synapses, it plays a crucial role in the maintenance of synaptic connections, since after the normal brain assembly is completed munc-18 knock-out mice neurons undergo apoptosis (Verhage et al., 2000). Therefore, maintaining synaptic connectivity may in part be regulated by differential effects that are exerted by neurotransmitters.

In the CNS the postsynaptic specializations develop prior to presynaptic specializations (Friedman et al., 2000; Ziv and Garner, 2001), relying upon reciprocal interactions between the synaptic partners. Initially, the motile dendritic filopodia that trigger presynaptic differentiation are devoid of PSD-95 clusters (Okabe et al., 2001). The earliest sign of postsynaptic specialization is the appearance of NMDA receptors and PSD-95 clusters (McGee and Bredt, 2003). PSD-95 clustering is followed by

accumulation of AMPA receptors (Friedman et al., 2000). Membrane bound molecules that are capable of organizing postsynaptic assembly at excitatory CNS synapses are EphB receptor tyrosine kinases. These receptors, when activated by their ligand ephrinB, bind and cluster NMDA receptors in cultured neurons (Dalva et al., 2000). Neuregulins are another class of molecules that play an important role in postsynaptic expression of neurotransmitter receptors. Specifically, transcripts of neuregulin isoforms are detected in developing presynaptic neurons and their proteins appear necessary for the expression of nAChRs during interneuronal synapse formation (Yang et al., 1998). Another protein that can cluster postsynaptic receptors is Narp (neuronal activity-regulated pentraxin). Narp is a secreted protein, a member of the pentraxin family and its expression is modulated by synaptic activity (Tsui et al., 1996). Narp induces AMPA receptor clustering and, when overexpressed in cultured spinal neurons, it increases the number of excitatory synapses (O'Brien et al., 1999). In addition, dominant-negative Narp expressed in axons suppresses AMPA receptor clustering, and the same effect is seen to a lesser extend when Narp is expressed in dendrites (O'Brien et al., 2002). However, modulating Narp expression that alters AMPA receptor clustering, does not perturb the assembly of presynaptic components (O'Brien et al., 2002), suggesting that presynaptic differentiation does not depend on Narp-induced clustering of AMPA receptors but that additional signals are required. Taken together, these studies serve to demonstrate that secreted factors such as neurotransmitters as well as membrane-bound molecules act on postsynaptic membranes to influence filopodial motility followed by the development of postsynaptic specializations. However, the exact order of events is not clearly defined yet.

Presynaptic differentiation

The earliest synaptic contact established by dynamic interactions between growth cone filopodia or neuronal processes play and inductive role in CNS synaptogenesis (Fiala et al., 1998; Jontes and Smith, 2000). The numbers of active and dynamic filopodial protrusions are inversely correlated with the development of stable dendritic spines and synapses (Dunaevsky et al., 1999; Jontes et al., 2000). These observations imply that dendritic action is deterministic for synapse formation (Ziv and Smith, 1996; Fiala et al., 1998; Jontes and Smith, 2000). Recently, axons have been shown to modulate synapse formation by regulating the motility of their filopodia. Axonal filopodia from cultured mossy fibers decrease their motility as their development proceeds, resulting in the stabilization of filopodia that contact their postsynaptic targets (Tashiro et al, 2003). After initial axo-dendritic contact, synapses can form within 1-2 hrs (Friedman et al., 2000; Okabe et al., 2001). Rapid recruitment of synaptic components that are already pre-assembled could be responsible for immediate synaptogenesis, avoiding the need to assemble the synapse from scratch. Cytoplasmic transport packets that contain some synaptic vesicle proteins and active zone components have been reported to travel along the developing axon (Ahmari et al., 2000; Zhai et al., 2001; Shapira et al., 2003). Once these packets reach the tip of the developing axon, they fuse with the plasma membrane, delivering the active zone components before the appearance of synaptic vesicle proteins necessary for exocytosis (Friedman et al., 2000). It has been proposed that these initially delivered active zone components function as a scaffold for synaptic vesicle proteins by either trapping preformed synaptic vesicle clusters (Ahmari et al., 2000; Friedman et al.,

2000) or by forming new synaptic vesicle clusters (Okabe et al., 2001). On the other hand, synaptic vesicles have also been demonstrated to contain proteins required for transmitter vesicle exocytosis besides the active zone components, suggesting that the mere fusion of these vesicles and their content with the presynaptic membrane is enough to establish active presynaptic sites (Shapira et al., 2003).

Cell adhesion molecules, which play very important roles in neuronal outgrowth and axon guidance, are also involved in triggering the assembly of synaptic specializations (Sanes and Yamagata, 1999; Brose, 1999; Tao and Poo, 2001; Benson et al., 2001; Garner et al., 2002). These molecules include members of the immunoglobulin (Ig) superfamily such as N-CAM/Fasciclin II, L1, sidekicks, nectin, and SynCAM (Schachner, 1997; Yamagata et al., 2002; Takai and Nakanishi, 2003; Biederer et al., 2002), Ca^{2+}-dependent homophilic cell adhesion proteins such as N-cadherins (Shapiro and Colman, 1999; Lee et al., 2001) and protocadherins (Frank and Kemler, 2002), the heterophilic cell adhesion proteins such as neurexins and neuroligins (Talmage and Role, 2004; Missler and Sudhof, 1998) and proteoglycans such as syndecans (Yamaguchi, 2002). Other synaptogenic cell interaction molecules that can provide signals for 'asymmetric' pre-and postsynaptic differentiation are the heterophilic adhesion molecules β-neurexins and their interacting counterparts, the neuroligins (Missler and Sudhof, 1998). Ectopic expression of neuroligins in non-neuronal cells *in vitro* induces presynaptic assembly in the contacting axon (Scheiffele et al., 2000). Overexpression of exogenous β-neurexins blocked this synaptogenic activity of neuroligins, suggesting presynaptic differentiation to be mediated by β-neurexins on the axonal plasma membrane. In support of this reasoning are the data where β-neurexins have been shown to be enriched at presynaptic terminals (Dean et al., 2003). For an overview of some of the components involved in central synapse formation, a schematic illustration of a mature glutamatergic synapse is provided in Figure 2.

Downstream effects of cell-cell contacts that inhibit neuronal outgrowth and induce synapse formation are not well understood. However, intracellular Ca^{2+} levels have been shown to rise within seconds of contact between pre- and postsynaptic cells (Dai and Peng, 1993; Zoran et al., 1993). It is therefore plausible that Ca^{2+} may provide a trigger for subsequent cellular and molecular changes which ceases neuronal growth and initiates synapse formation (Rehder et al., 1996). Consistent with this notion is the evidence that Ca^{2+} does indeed regulate several aspects of neuronal development such as neurite outgrowth (Anglister et al., 1982; Cohan and Kater, 1986; Mattson and Kater, 1987; Streit and Lux, 1989,1990) and growth cone motility (Kater et al., 1988; Lipton and Kater, 1989; Kater and Mills, 1991; Rehder and Kater, 1992; Davenport and Kater, 1992). Depending on the neuronal compartment and environmental clues, rises in intracellular Ca^{2+} levels have different effects, ranging from regulation of transcription to growth cone turning (Spitzer, 2002). Since in most cases increase in Ca^{2+} levels upon cell-cell contact is related to suppression of growth, it is thought that a rise in intracellular Ca^{2+} concentrations functions as a "stop" signal for the elongating neurites (Kater et al., 1988; Kater and Mills, 1991; Fields et al., 1993; Kater and Rehder, 1995). Based on other studies there appears to exist a window of action for the intracellular Ca^{2+} concentration in which Ca^{2+} mediates neuronal growth. Concentrations either above or below that window inhibit neuronal growth. For instance, lack of synaptic activity or a large increase in intracellular Ca^{2+} concentrations in filopodia causes shrinkage, collapse and

elimination of spines, whereas a moderate elevation of intracellular Ca^{2+} levels causes elongation of both existing spines and the formation of new ones (Segal, 2001). The development of Ca^{2+} 'hot spots' between neurons has also been demonstrated during synaptogenesis (Feng et al., 2002). Taken together, it is clear that Ca^{2+} is one of the main players in a number of processes ranging from neuronal outgrowth, neurite elongation to growth cone turning and synapse formation. However, neither the nature of molecules that alter Ca^{2+} homeostasis in the presynaptic cell nor the underlying mechanisms have been identified.

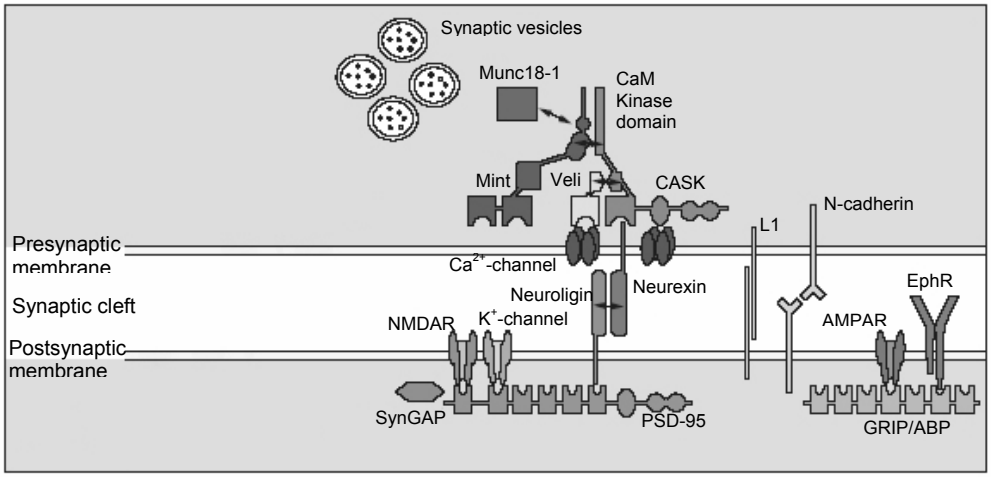

Figure 2: Synapse formation at Central Synapses.
A schematic overview of glutamatergic synapses and some of their components in the adult brain. (Figure is modified from Cantallops and Cline, 2000). Ca^{2+} channels and NMDA receptors cluster at the pre-and postsynaptic sites respectively. Neurexin (presynaptic) and neuroligin (postsynaptic) interactions induce clustering of various components of the synaptic machinery.

1.1.4. Neurotrophins and synapse formation.

In addition to the cell-cell signaling that brings about specific changes in the synaptic machinery of partner cells, a variety of extrinsic factors also influence the development of synaptic structures during synapse formation. Several diffusible factors including the secreted signaling proteins Wnts, neurotrophins, neuregulins and CNS agrin have been identified. A number of these not only promote neuronal outgrowth but also exert pronounced effects on synapse formation. Neurotrophins are secreted, diffusible factors that can prime neurons over a distance for synapse formation. Neurotrophins such as nerve growth factor (NGF), neurotrophin-3 (NT-3), NT-4/5 and brain-derived neurotrophic factor (BDNF) are known to promote synaptogenesis, besides their role in neuronal survival and differentiation (Bonhoeffer, 1996). In addition, these neurotrophins have rapid and long-term effects on synaptic strength during development and at mature synapses. For instance, they may either enhance or suppress inhibitory or excitatory synaptic transmission through their actions either at the pre-or postsynaptic sites (Schuman, 1999). BDNF influences a number of processes such as dendritic and axonal arborization and increases synapse number (McAllister et al., 1999; Alsina et al., 2001),

which facilitates the development and maturation of both inhibitory and excitatory synaptic circuits in cultured neurons (Vicario-Abejon et al., 1998; Bolton et al., 2000; Marty et al., 2000) and cerebellum inhibitory synapses (Seil and Drake-Baumann, 2000). BDNF-coated beads induce localized neurotransmitter release at the contact sites on the developing axon (Zhang and Poo, 2002). These studies show that this particular neurotrophin exert its effect on both pre-and postsynaptic neurons. NT-3 has been shown to exert different effects on developing neurons in the sensory system. In recent experiments in mice, the coding region of BDNF was replaced with that of NT-3 (Agerman et al., 2003). These mutant mice showed that BDNF was required for proper innervation and synapse formation in the vestibular system, whereas NT-3 was sufficient for vestibular ganglion neuron survival. In addition, NT-3 could not replace BDNF in the gustatory system. These studies demonstrate that different neurotrophins promote different processes at various developmental stages in the nervous system indicating the importance of temporal and spacial expression patterns of neurotrophins and their receptors in synapse formation.

Like neurotrophins, secreted Wnt proteins also play a role at different stages of development, including synaptogenesis. Wnt-7a released form granule cells in the cerebellum has been shown to induce axon growth cone remodeling of mossy fibers, followed by growth cone clustering of synapsin I, a synaptic vesicle-associated protein (Hall et al., 2000). In Wnt-7a deficient mice, synapse formation in the cerebellum, although ultimately proceeds normally, it is nevertheless, initially delayed. Similarly, Wnt-3, which is secreted by the dendrites of spinal cord motor neuron, also promotes terminal arborizations of sensory neuron axons by retrograde signaling. Like neurotrophins, special and temporal activation of different Wnt signaling pathways could contribute to the specificity of synaptic connections.

The above studies serve to demonstrate that a variety of intrinsic and extrinsic factors may bring about both short and long-term changes in synaptic partners during synapse formation. In addition, it is clear that preassembled synaptic packets can be transported to the presynaptic terminal and that synaptic proteins are delivered to the postsynaptic terminal to provide neurons with the necessary materials to form synapses. In both processes the origin of the proteins and the preassembled packets is the soma. However, strong evidence exists for the synthesis of proteins locally in the growth cone and during the formation of synapses. Therefore, the exact contribution of local protein synthesis at, and the transport of proteins from the soma to the synaptic sites for the formation of synapses has yet to be elucidated.

1.2. Non-somal Protein Synthesis and its requirement for synapse formation and function.

It has long been debated whether proteins required for synapse formation, maintenance and synaptic plasticity are synthesized in the somata and transported to distally located synapses or whether their synthesis occurs *de novo* in the extrasomal compartments. The evidence that most house keeping proteins essential for axonal viability and synaptic function can be synthesized locally came originally from experiments on *Procambarus clarkii* (Crayfish). In most mammalian preparations, the axotomized neurons undergo rapid degeneration after the removal of their somata (Kromer, 1987; van der Zee and Hagg, 2002). However, some crustacean neurons not only remained viable for spike

propagation but were also synaptically and functionally "alive" months after the removal of their somata (Atwood et al., 1973; Hoy, 1969; Hoy et al., 1967; Wine, 1973, Krasne and Lee, 1977). These observations raised the following questions. What is the half-life of any given synaptic protein? Are proteins required for synapse maintenance and function synthesized locally from the mRNA that are located in the extrasomal compartment? What is the half-life of various mRNA species? An interesting study conducted a decade later demonstrated that axons severed from their somata not only survived for months, but also remained synaptically and functionally viable. This long-term survival of axons was attributed to glia cells, which "donated" their nuclei to the served axons thus allowing them to manufacture proteins. Specifically, after several months of soma removal, the membranes around the invading cells had disappeared, leaving behind apparent functional multiple nuclei (Atwood et al., 1989). These data suggested that in rock lobsters the axotomized axons can survive for several months following the invasion of satellite cells and subsequent 'donation' of glia specific organelles that now sub-serve neuron specific functions. Similarly, axonal segments of *Aplysia* neurons in culture preserved their morphological integrity and physiological properties for up to 14 days and maintained their passive and excitable membrane functions in the absence of *de novo* protein synthesis (Benbassat and Spira, 1993). Interestingly, the *Aplysia* transected axonal segments survived twice as long when cultured in physical contact with intact homologous neurons. In addition to their morphological and electrophysiological viability, the severed axons also extended neurites, maintained normal passive and excitable membrane properties, formed gap junctions with the intact neurons and maintained normal free intracellular Ca^{2+} levels (Benbassat and Spira, 1994). Since the long term survival of these axonal segments depended on physical contact with intact neurons, this survival was attributed to direct transfer of material from the intact neurons to the transected axonal segments. Transfer of proteins from neurons and glial cells to axotomized axons had been shown previously in squid (Gainer et al., 1977; Lasek et al., 1977) and crayfish (Meyer and Bittner, 1978a,b; Tytell et al., 1986; Sheller and Bittner, 1992). Taken together, it appears safe to infer that in the above mentioned examples, the axons severed from their somata can survive for long periods of time through the glia-transfer mechanisms.

Indirect evidence for axonal ability to synthesize proteins locally came from experiments, which demonstrated that constituents of the translation machinery, tRNAs, may be present in axons (Black and Lasek, 1977). Later, rRNA was detected in this neuronal compartment as well (Giuditta et al., 1980). In the squid giant axon other components of the protein synthetic machinery such as eukaryotic initiation and elongation factors, aminoacyl-tRNA synthetase, and ribosomes (Giuditta et al., 1977, 1980, 1991; Sotelo et al., 1999) have been detected, not only in the axon but also in presynaptic endings of retinal photoreceptor neurons (Crispino et al., 1997; Martin et al., 1998). Similarly in *Lymnaea* neurons the presence of ribosomes in axons, growth cones and varicosities has been demonstrated (Van Minnen, 1994b; Van Minnen et al., 1997). Besides the translational machinery, a population of transcripts encoding for proteins also appear to reside in the axonal compartment of neurons. Messenger RNAs have first been detected and identified in the molluscan model system. The squid giant axon harbors a diverse population of mRNAs that encode for proteins such as β-tubulin, β-actin and kinesin that build the cytoskeleton (Gioio et al., 1994). Transcripts encoding for

neuropeptides have been identified in *Aplysia* and *Lymnaea* axons (Van Minnen et al., 1988; Landry et al., 1992; Van Minnen, 1994a). The different mRNA species that encode for proteins ranging from cytoskeletal proteins such as β-actin (Olink-Coux and Hollenbeck, 1996) to odorant receptors (Ressler et al., 1994), to secretion hormones such as oxytocin and vasopressin have been detected in the extrasomal compartments (Mohr et al., 1991).

The presence of various translation machinery components and mRNAs does not automatically prove that extrasomal compartments indeed synthesize proteins locally. Convincing evidence was provided by Giuditta et al., (1968) in the isolated squid giant axon, which rapidly incorporated radio-labeled amino acids into proteins, and this process was inhibited by ribosome-based protein synthesis inhibitors (Giuditta et al., 1968). These authors furthermore incubated the giant axon with [^{35}S]-methionine for 1 hour before extracting the axoplasm from the distal end of the giant axon. They showed that the polyribosomes that were present in the axoplasm contained radiolabeled polypeptides (Giuditta et al., 1991). These data thus demonstrated that the giant axon does indeed contain ribosomes which actively synthesize proteins. These studies so far have demonstrated axonal capability of local protein synthesis, but were not conclusive on the identity of the proteins that were synthesized locally. To demonstrate that axons are able to synthesize secretory and integral membrane proteins, Van Minnen et al., (1997) and Spencer et al. (2000a) have used similar approaches in *Lymnaea* neurons. Isolated *Lymnaea* axons injected in culture with a mRNA encoding the peptide precursor for the egg laying hormone (ELH), translated this transcript into it's cognate protein (Van Minnen et al., 1997). Furhtermore, injection of an mRNA encoding a G-protein coupled receptor into isolated *Lymnaea* axons resulted in local translation as well as functional membrane integration of this receptor in isolated axons (Spencer et al., 2000a).

The significance of local protein synthesis has been demonstrated by studies focusing on synaptic plasticity. In addition to a variety of extrinsic factors that can influence synaptic sites to modulate the efficacy of synaptic transmission over a longer time window, local protein synthesis seems to be the intrinsic mechanism which may also alter the mode of neuronal communication. For instance, in rat hippocampal Schaeffer collateral synapses neurotrophins can induce long-term synaptic potentiation, that relies on local protein synthesis (Kang and Schuman, 1996). Similarly, in *Aplysia* compartment-specific long-term synaptic plasticity at sensory-motor neuron synapses has been shown to be dependent on the local translation of proteins (Martin et al., 1997; Casadio et al., 1999; Sherff and Carew, 1999). Furthermore, axons severed from their cell bodies were shown to require protein synthesis for the formation of synapses (Schacher and Wu, 2002). Besides this protein synthesis dependency of neurons for synaptic plasticity, in developing axons growth cone reponses to various guidance cues are also dependent on local protein synthesis (Campbell and Holt, 2001). Therefore, it appears that both in early development as well as in plasticity of "mature" synapses, local protein synthesis is critical for their adaptation to external cues, although the underlying mechanisms remain to be determined.

Taken together, the introduction so far has described studies that provide an overview illustrating that the mechanisms underlying synapse formation in the nervous system are complex. Although some fundamental principles governing synapse formation at the NMJ apply to central synapse formation, it is however evident that both the

molecules and the underlying mechanisms for CNS synapse formation are partly different. Synapse formation seems to require proteins from the cell soma and proteins that are synthesized locally. Moreover, contacts between synaptic partners bring about specific changes in the synaptic structure of both partner cells, a process that is also influenced by extrinsic factors, such as the growth factors. Thus, both the cellular and molecular mechanisms that determine the specificity of target cell selection and specific synapse formation in the nervous system remain poorly defined. This lack of fundamental knowledge in our basic understanding of synapse formation is due to fact that cell-cell interactions between defined sets of pre-and postsynaptic neurons can only rarely be investigated reliably in complex mammalian systems.

1.3. Lymnaea stagnalis neurons; A Model System Approach towards revealing novel mechanisms in synapse formation

Studies on both vertebrates and invertebrates have revealed that various fundamental mechanisms of neurodevelopment are highly conserved in a wide variety of species. However, a variety of molluscan species is unique in that their individually identifiable cells are amenable for direct cellular and synaptic analysis at a resolution that is unapproachable in most other species. These neurons can also be extracted from the intact brain and grown in cell culture. In addition, multiple neurons plated together in cell culture regenerate their neurites, find their specific target and form appropriate synapses (Bulloch and Syed, 1992; Fernández-de-Miguel, 1997; Haydon and Drapeau, 1995; Munno and Syed, 2003; Ready and Nicholls, 1979). Among other invertebrate neurons, *Lymnaea* neurons are large in size and easily identifiable on the basis of their size, color, position and function. The best described is a neuronal network underlying respiratory behavior. This network is comprised of three neurons; the right pedal dorsal 1 (RPeD1), the visceral dorsal 4 (VD4), and the input 3 interneuron (IP3I). The individual synapses between these neurons are well characterized. The respiratory central pattern generator (CPG) neurons can also be isolated in cell culture where they not only recapitulate their specific pattern of synaptic connectivity but also generate patterned rhythmic activity which is similar to that seen *in vivo* (Syed et al., 1990). In addition to neurite-neurite synaptogenesis, synapses between *Lymnaea* neurons can also be reconstructed in a soma-soma configuration (Feng et al., 1997). The soma-soma synapses are also target cell contact specific and require gene transcription and *de novo* protein synthesis. The formation of specific excitatory but not the inhibitory synapses between soma-soma paired *Lymnaea* neurons requires extrinsic trophic factors and these effects are mediated through receptor tyrosine kinases (Hamakawa et al., 1999). Subsequent studies showed that the neurotrophic factor-induced effects involved the modulation of postsynaptic nicotinic ACh receptors (Woodin et al., 2002). Specifically, in the absence of trophic factors, the RPeD1 and VD4 formed mutual inhibitory synapses which do normally not exist in the brain. However, increasing concentrations of trophic factors bring in an excitatory component to the VD4->RPeD1 synapse (Woodin et al., 2002).The development of this excitatory component required RTK activation in RPeD1, and not in VD4. Since the neurotransmitter used by VD4 is acetylcholine, both excitatory and inhibitory components of the synaptic responses were blocked by specific cholinergic antagonists (Woodin et al., 2002). Exogenous application of ACh to single LPeD1 and RPeD1 neurons (both function as postsynaptic cells) also demonstrated the appearance of

excitatory responses in the presence of trophic factors, further suggesting that a CM-induced switch from inhibitory to excitatory responses may just involve the postsynaptic cell. Taken together, these studies have provided valuable information regarding the mechanisms of both inhibitory and excitatory synapse formation between two specific cell pairs.

1.4. The aim of this study.

From the introduction it is clear that many factors play an important and crucial role in the formation of synapses between specific neurons. Lymnaea neuronal preparations have simplified the study of synapses greatly in that it has allowed us access to and study of single synapses. In particular, soma-soma cultured neurons from *Lymnaea* bypasses the role of axons in synapse formation and gave the possibility to study synapse formation directly. Since in the intact brain axons are the neuronal compartments that actually synapse onto other neurons, the chapters in this study are focused on the role of extrasomal compartments in different aspects of synapse formation and the maintenance of axonal compartments.

To this end, I have taken VD4 and LPeD1 as the model system and investigated the role of isolated axons in the maintenance of existing synapses. In addition, I have studied the role, or requirement, of somata and axons in the formation of new synapses. Furthermore, the role of external factors and intracellular processes in the maintenance of the isolated axonal compartments has been elucidated. Finally, I have investigated the necessity of neurotransmitter-receptor interactions as well as the roles of the different neuronal compartments in the formation and specificity of synapses.

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chapter

2

Specific Synapses Between *Lymnaea* Neurons Re-establish In The Soma-Axon Configuration

with David W Munno and Naweed I Syed

Abstract

The formation of specific synapses in the brain is central to all animal functions in adult animals, however the precise mechanisms remain largely unknown. Because cell-cell interactions between individual pre-and postsynaptic neurons are often difficult to study directly, a variety of cell culture approaches have been used to define fundamental principles of synaptogenesis. To elucidate the mechanisms of synapse formation between *Lymnaea* neurons, in this study we attempted to reconstruct specific synapses between somata and their isolated axons. We provide direct evidence that specific, excitatory synapses between visceral dorsal 4 (VD4) and its postsynaptic partner left pedal dorsal 1 (LPeD1) reform in a soma- axon configuration. The soma-axon synapses were target cell-contact specific and were similar to those seen both *in vivo* and *in vitro*. Moreover, we demonstrate that the axons severed from their respective somata, after the formation of appropriate synapses, can maintain these synapses in the absence of their cell bodies. However, the pre- but not the postsynaptic soma was found to be required for new synapse formation.

Introduction

To establish the precise synaptic connectivity that forms the basis of neural network organization and function in the adult brain, developing neurons must extend their axonal and dendritic processes (i.e. growth cones) towards their potential target cells. Following target cell recognition, neurite outgrowth is terminated and synapses begin to develop. It is generally accepted that, prior to contacting their synaptic partners, both pre- and postsynaptic elements are ready for synaptic transmission (Haydon and Drapeau, 1995). For instance, recent studies have shown that various pre- (Ahmari et al., 2000; Shapira et al., 2003) and postsynaptic (O'Brien et al., 1997; Rao et al., 1998; Levi et al., 1999; Prange and Murphy, 2001; Marrs et al., 2001) components of the synaptic machinery may be pre-assembled in the form of synaptic 'packets' prior to target cell contact. Upon contact, these 'ready made' synaptic components can be dispatched immediately to designated synaptic sites, thus allowing a 'fast-track' synaptogenic program to proceed in the absence of gene transcription and new protein synthesis (Munno and Syed, 2003). These studies thus, suggest that various proteins required for synaptic programs are most likely present in the extrasomal compartments (i.e. axons and dendrites) and that the synaptogenesis may proceed in the absence of somata based signaling.

To decipher the precise contributions of pre- and postsynaptic somata in synapse formation, we have attempted to reconstruct synapses between the isolated axons of identified *Lymnaea* neurons. Axons severed immediately after neuronal isolation, were juxtaposed in cell culture and synapses were tested electrophysiologically. We provide evidence that existing synapses can be maintained for several days between isolated axons. However, for new synapse formation, only the presynaptic and not the postsynaptic cell body, is required.

Materials and Methods

Animals. Lymnaea stagnalis were maintained at room temperature in a well-aerated aquarium containing filtered water. For experiments involving cell isolation, snails approximately 1-2 months old (shell length 18-20 mm) were used, while conditioned medium (CM, see below) was prepared from 2-3 month old animals (shell length 25-30 mm).

Cell Culture. Neurons were isolated from the central ring ganglia and maintained in cell culture as described previously (Syed et al., 1990; Ridgway et al., 1991; Syed et al., 1999). Briefly, snails were anesthetized with 10% Listerine solution (ethanol, 21.9%; methanol, 0.042%) in normal *Lymnaea* saline [(in mM): 51.3 NaCl, 1.7 KCl, 4.0 $CaCl_2$ and 1.5 $MgCl_2$] buffered to pH 7.9 with HEPES. The central ring ganglia were then washed several times (3 washes, 15 min each) with normal saline containing antibiotic (gentamycin, 50 μg/ml). The central ring ganglia were then treated with enzyme (trypsin) followed by enzyme inhibitor (trypsin inhibitor) and pinned down at the bottom of a dissection dish. All procedures were performed under sterile culture conditions.

CM was prepared by incubating gentamycin (20 μg/ml)-treated ganglia in Sigmacote (Sigma, St. Louis, Mo.) -treated glass petri dishes, containing defined medium (DM, L-15; Life Technologies, Gaithersburg, MD; Special Order). DM consisted of serum free, 50% L-15 medium with added inorganic salts (in mM: 40 NaCl, 1.7 KCl, 4.1 $CaCl_2$, 1.5 $MgCl_2$, and 10 HEPES, pH 7.9) and 20 μM gentamycin. The ganglia were incubated in a humidifier for 3 - 4 days (Syed et al., 1999; Wong et al., 1981) and the resulting CM was frozen (-20^{o} C) until used.

The identified neurons were isolated by applying gentle suction through a fire-polished, Sigmacote-treated pipette. The isolated neurons were then plated on poly-L-lysine-pretreated glass coverslips (Ridgway et al., 1991) in either DM or CM. Axons were isolated by first plating the cell body along with its intact axon segment in cell culture and allowing it to adhere to the poly L-lysine coated dish. The axon was then immediately severed from the cell body by using a sharp glass pipette, and the severed cell body was subsequently removed from the culture dish. Soma-axon synapses were prepared by juxtaposing the soma to the isolated axon. Axon-axon synapses were prepared by juxtaposing the axon segments of the identified neurons, followed by removal of both somata.

Electrophysiology. Neuronal activity was monitored using conventional intracellular recording techniques, as described previously for *Lymnaea* (Syed and Winlow, 1991). Glass microelectrodes (1.5 μm internal diameter; World Precision Instruments, Sarasota, FL) were filled with a saturated solution of K_2SO_4 (resistance, 20-40 MΩ). An inverted microscope (Axiovert 135; Zeiss, Thornwood, NY) was used to view the neurons, which were impaled using Narashige (Tokyo, Japan) micromanipulators (MM202 and MM 204). Amplified electrical signals (Neuro Data Instrument Corp.) were displayed on a digital storage oscilloscope (PM 3394; Philips, Eindhoven, The Netherlands) and recorded on a chart recorder (TA 240S; Gould, Cleveland, OH).

Chemicals.
Trypsin, trypsin inhibitor, and Poly-L-Lysine were obtained from Sigma.

Results

Specific synapse formation between VD4 and LPeD1 somata
To test whether specific synapses between *Lymnaea* neurons VD4 and LPeD1 reform in cell culture in a soma-soma configuration, identified neurons were isolated from their respective ganglia and paired in culture in a soma-soma configuration. After 24hrs of cell culture, synapses were tested electrophysiologically. Specifically, both pre-and postsynaptic neurons were impaled simultaneously with sharp electrodes. Induced action potentials in VD4, elicited 1:1 excitatory postsynaptic potentials (EPSPs) in LPeD1, which were similar to those seen *in vivo* (Hamakawa et al., 1999). Figure 1A shows a VD4-LPeD1 soma-soma pair, with an example of their typical chemical synapse depicted in Figure 1B (n=5). Specifically, induced action potentials in the presynaptic soma (at arrows) evoked 1:1 excitatory postsynaptic potentials (EPSPs) in the postsynaptic cell (Figure 1B). These data confirm previous studies that specific synapses between *Lymnaea* neurons reform in a soma-soma configuration and set the stage for further studies on defining the precise role of the presynaptic soma in synaptogenesis.

Synapse formation between VD4 and LPeD1 in the axon-axon configuration.
Because soma-soma synapses do normally not occur *in vivo*, the functional significance of this model remains debatable. Therefore, to test whether synapses between VD4 and LPeD1 also reform between the neurites, neurons were isolated from their respective ganglia and their axons were overlaid (Figure 2A - *upper panel*). After 18-24 hrs of cell culture, neurons were impaled with sharp electrodes and intracellular recordings were made as described above. In all preparations, action potentials in VD4 (at arrows) induced reliably 1:1 EPSPs in LPeD1 (n=5) (Figure 1C), which were similar to those seen in a soma-soma configuration. These data thus demonstrate further the usefulness of soma-soma and neurite-neurite preparation for ongoing studies on synapses formation.

Synapses between VD4 and LPeD1 axons are maintained in the absence of their somata.
To provide direct evidence (i.e. in the absence of other neurons and glia) that synaptic transmission between axons severed from their respective somata remains functional, synapses were examined in cell culture. Specifically, VD4 and LPeD1 neurons were juxtaposed such that their respective axon stumps overlaid each other (Figure 2A - *upper panel*). On day 1 (after 24 hrs), intracellular recordings revealed excitatory synapses between the pairs (n=6) (Figure 2A). To test for axonal ability to maintain these synapses in the absence of their respective somata, both VD4 and LPeD1 axons were subsequently severed and their somata removed (Figure 2A – *lower panel*). VD4 and LPeD1 axons were impaled with sharp intracellular electrodes and synapses were re-examined electrophysiologically 24 hours after the soma removal (day 2). Both spontaneous and induced action potentials in VD4 axon generated 1:1 EPSPs in LPeD1 axon (n=6) (Figure 2B). The ability of isolated axons to maintain synaptic transmission was independent of

whether VD4 (n=5) or LPeD1 soma (n=6), or both (n=6) were removed (Figure 3). Although the efficacy of synaptic potentials recorded from severed axons did not change significantly on day 2 (mean EPSP amplitude day 1 = 10.0 ± 3.8 and day 2 = 8.5 ± 1.5 mV), a significant reduction in the EPSPs amplitude was however, observed on day 3 (5.9 ± 0.2 mV, $p < 0.01$) (Figure 3). These data thus demonstrate that the isolated axons can indeed maintain synapses in culture for several days in the absence of their somata, however, the efficacy of synaptic transmission tends to be reduced over time.

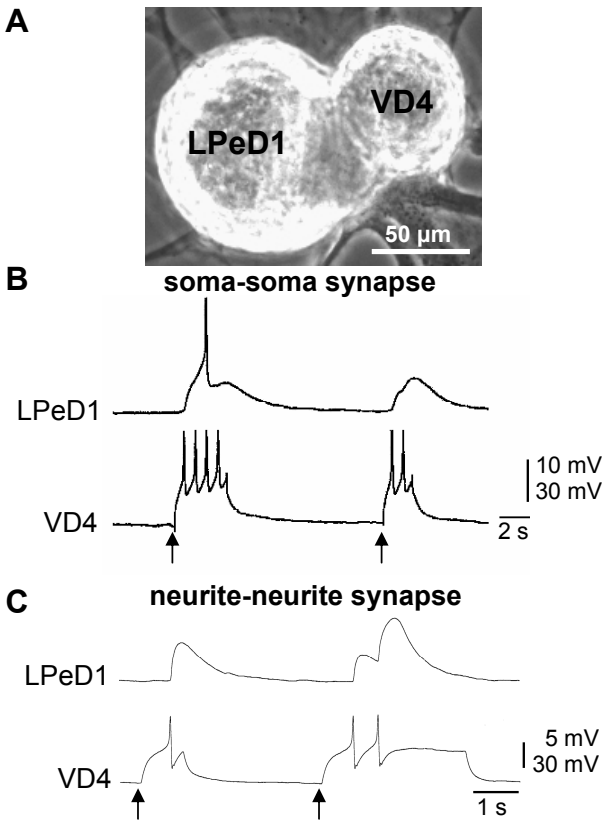

Figure 1: The soma-soma synapse.

(**A**) The presynaptic (VD4) and postsynaptic neuron (LPeD1) form excitatory synapses when paired in a soma-soma configuration. (**B**) Action potentials in VD4 (at black arrows) induced 1:1 excitatory postsynaptic potentials (EPSPs) in the LPeD1. (**C**) **The neurite-neurite synapse**. Isolated neurons VD4 and LPeD1 were cultured in CM with their axon stumps overlaid. After 18-24 hrs of cell culture, neurons were impaled with sharp electrodes and simultaneous intracellular recordings were made to test synapses. Induced action potentials in the VD4 (at black arrows) generated 1:1 EPSPs in LPeD1 (n=5), indicating that excitatory synapses also develop in the neurite-neurite configuration.

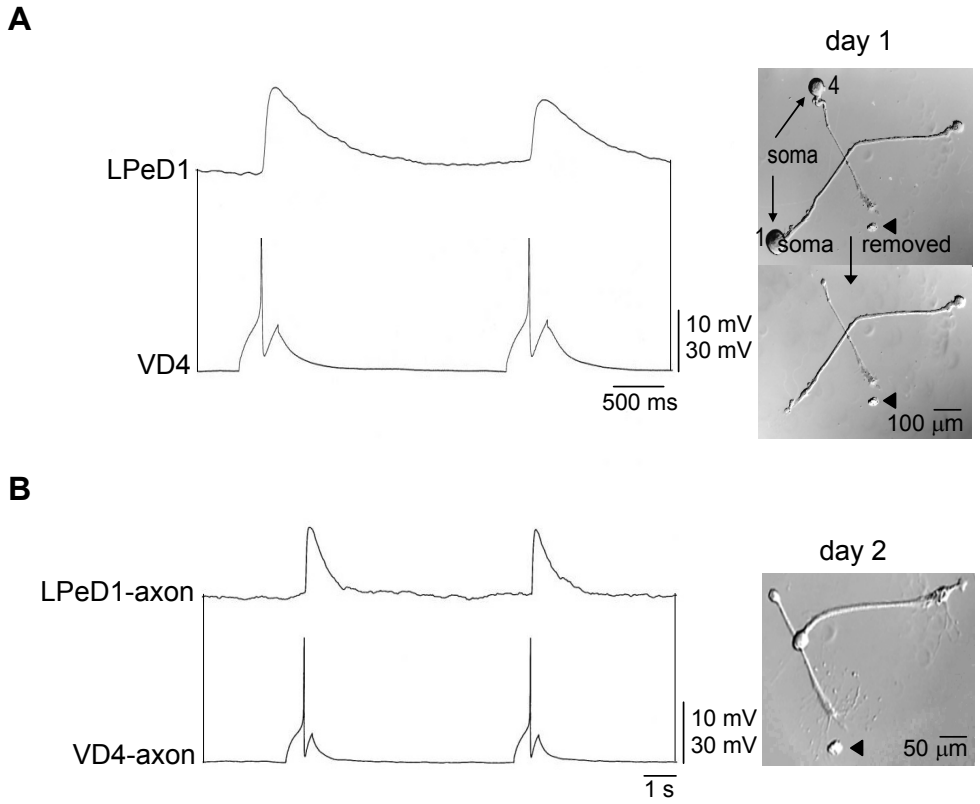

Figure 2: Specific synapses between *Lymnaea* axons are maintained in cell culture.

(**A**) VD4 and LPeD1 were paired in CM (insert - upper panel), such that their axons overlaid each other. After 12-18 hours, synapses were tested electrophysiologically. Induced action potentials in VD4 generated 1:1 excitatory postsynaptic potentials (EPSPs) (n=6). Following the demonstration of an excitatory synapse, either VD4 or LPeD1 axon, or both axons (insert - lower panel) were severed and their cell bodies were removed from the culture dish. (**B**) 12 –24 hours after soma removal (insert), the excitatory synapse remained intact and action potentials in VD4 continued to elicit 1:1 EPSPs in LPeD1 (n=6).

Presynaptic but not the postsynaptic soma is required for excitatory synapse formation between VD4 and LPeD1.

To determine the involvement of VD4 and/or LPeD1 somata in the formation of new synapses, severed axons were tested for their ability to establish new synapses in the absence of their somata. Axons and somata were paired either in a soma-axon or axon-axon configuration. Specifically, either VD4 soma or its severed axon was juxtaposed against LPeD1 somata or its severed axon. Synapses were tested electrophysiologically after 12-24 hours. Pairing of VD4 and LPeD1 axons did not result in synapse formation between the axons. That is, induced action potentials in the presynaptic axon (Figure 4A)

failed to generate electrophysiologically detectable responses in postsynaptic axons (n=13). Similarly, pairing of severed axon from VD4 with LPeD1-soma also did not result in synapse formation (n=10) (Figure 4B). However, when the VD4 soma was paired with the LPeD1 axon (Figure 4C), excitatory synapses did form whereby induced action potentials in VD4 soma generated 1:1 EPSPs in LPeD1 axon (Figure 4D). These data demonstrate that: 1) Axons severed from postsynaptic neurons are capable of synaptogenesis, and 2) the presynaptic but not postsynaptic soma is required for synapse formation.

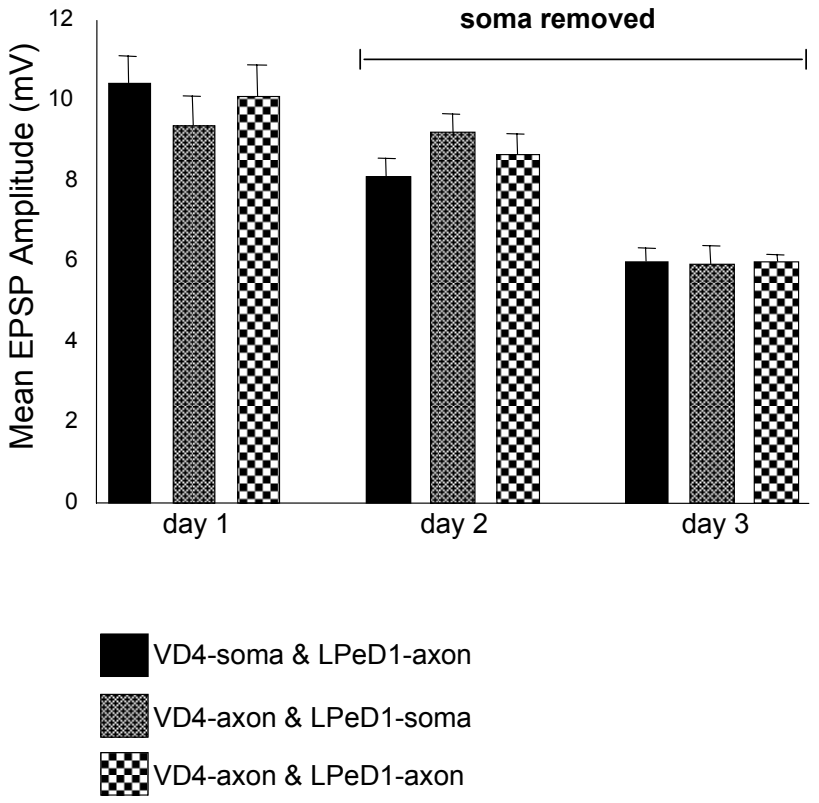

Figure 3: Summary of synaptic transmission between somata and severed axons of VD4 and LPeD1. Summary data showing the efficacy of synaptic transmission between somata and severed axons of VD4 and LPeD1. Synapses were tested electrophysiologically on Day 1 and either one or both somata were removed and synapses re-tested on Day 2 and Day 3. For VD4 axon/LPeD1 soma pairs the mean EPSP amplitude was 9.3 ± 2.1 mV on day 1, 9.1 ± 1.4 on day 2, and 5.9 ± 0.9 mV on day 3. For VD4 soma/LPeD1 axon pairs the mean EPSP amplitude was 10.4 ± 2.7 mV on day 1, 8.0 ± 1.3 on day 2 and 5.9 ± 0.7 mV on day 3. For the axon-axon pairs the mean EPSP amplitude was 10.0 ± 3.8 mV on day 1, 8.6 ± 1.5 on day 2 and 5.9 ± 0.2 mV on day 3. Although the efficacy of synaptic strength on day 2 was similar to that recorded on day 1 (with the exception of the VD4 soma/LPeD1 axon pair), a significant reduction was observed on day 3 ($p < 0.01$ for all pairs).

Synaptic specializations at the presynaptic site of soma-axon pairs.
Previous studies have shown that Ca^{2+} hotspots develop at the contact site between soma-soma paired neurons, which are target cell and contact site specific (Feng et al., 2002). To demonstrate further that the presynaptic secretory machinery between soma-axon pairs is also specialized at the contact site, functional presynaptic sites were labeled with the fluorescent dye FM1-43 by active endocytosis. Specifically, the VD4 neurons were paired with the LPeD1 axon overnight (Figure 5A), and the next day the presynaptic cell

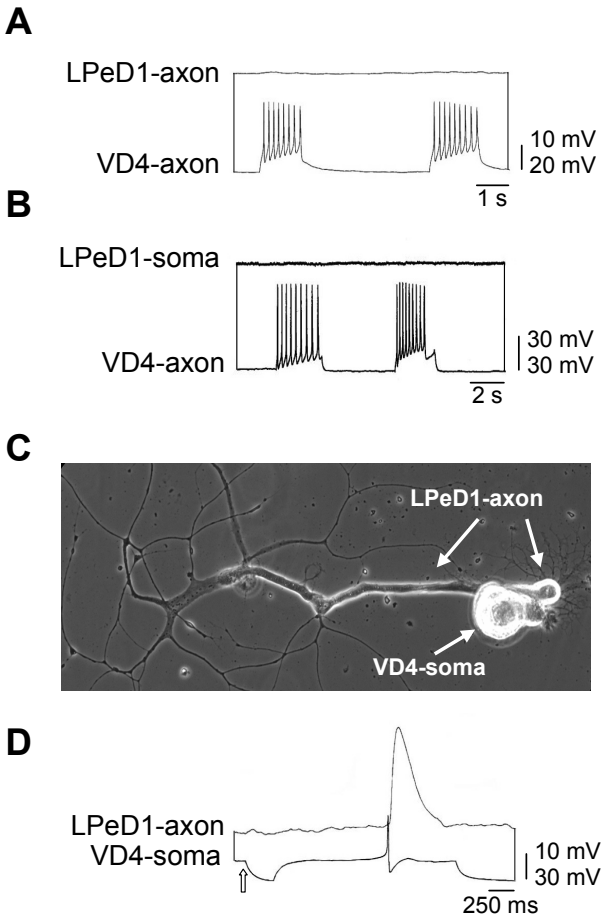

Figure 4: Presynaptic cell body is required for excitatory synapse formation in cell culture.
Axon-axon or soma-axon pairs were maintained overnight in CM, and synapses were tested electrophysiologically. (**A**) When the severed VD4-axon was paired either with the LPeD1-axon (n=13) or (**B**) its cell body (n=10), no excitatory synapses were detected. Specifically, action potentials in VD4 axons did not induce postsynaptic responses in either (**A**) LPeD1 axon or (**B**) its soma. (**C**) When VD4-soma was paired with LPeD1-axon, appropriate excitatory synapses were detected. (**D**) Induced action potentials in VD4 produced 1:1 EPSPs in LPeD1 axon. Hyperpolarizing pulses generated in VD4 (at open arrow) did not pass into LPeD1 suggesting that the synaptic transmission is chemical.

was impaled with an intracellular electrode. Following the intracellular impalement, the dye FM1-43 (8-30 μM bath concentration) was added to the bath and images were acquired. VD4 was then induced to fire action potentials (up to 10 action potentials) in the presence of the dye. FM1-43 was then washed away with fresh DM and images were

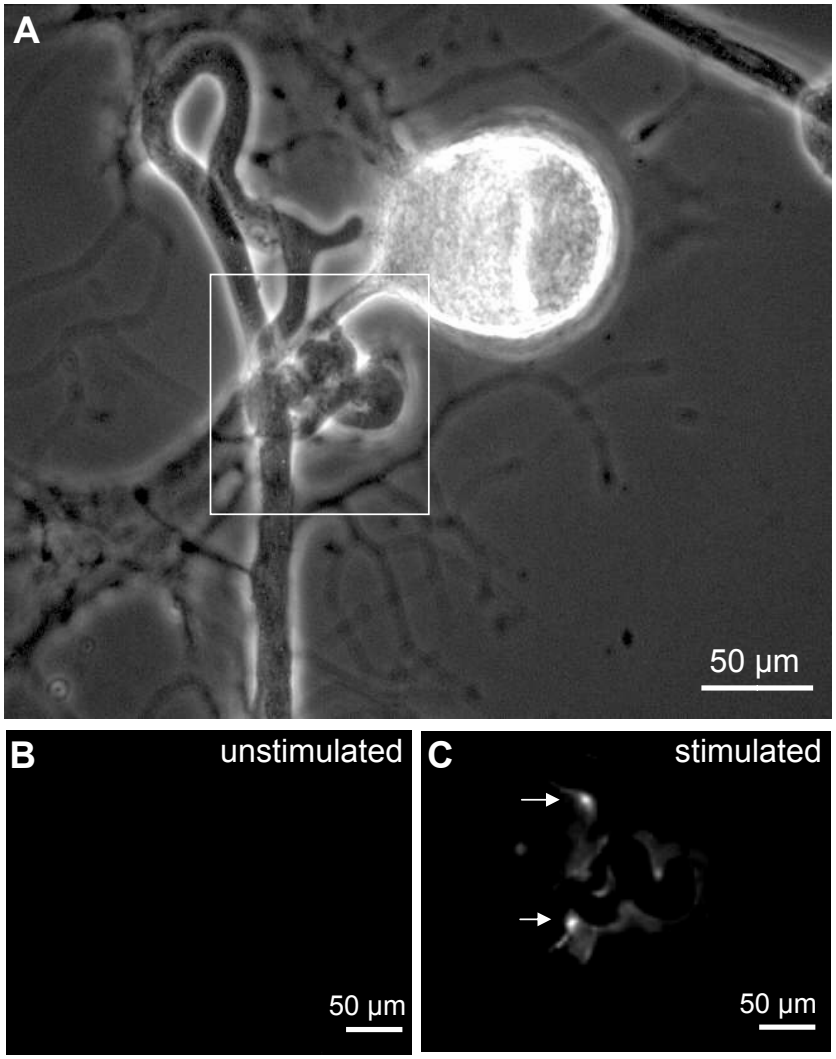

Figure 5: Synaptic uptake of FM1-43 in the soma-axon preparation.
(**A**) Soma-axon pairs were cultured overnight and loaded with the fluorescent dye FM1-43. (**B**) Unstimulated soma-axon pairs demonstrate no detectable dye uptake, whereas (**C**) stimulated soma-axon pairs exhibit specific fluorescent staining (white arrows) at the site of contact between VD4 soma and LPeD1 axon.

acquired again. The unstimulated cell did not exhibit any staining at the contact site (Figure 5B), whereas fluorescently labeled puncta were clearly discernable at the contact site between the soma-axon pairs (Figure 5C). These results demonstrate that the contact site between the soma-axon pairs exhibit specialized sites for exocytosis and endocytosis and are thus physiologically functional synaptic sites.

Discussion

In this chapter it has been demonstrated that excitatory synapses similar to those seen *in vivo* re-form *in vitro* between the identified neurons VD4 and LPeD1 in a soma-soma as well as a neurite-neurite configuration. Furthermore, these specific synapses were maintained in the absence of pre-and postsynaptic somata. However, for new synapse formation the pre- but not the postsynaptic soma was required.

To unravel the the mechanisms of synapse formation in the nervous system, several models have been utilized to obtain valuable information regarding the synaptogenic program. For instance, studies of the neuromuscular junction (NMJ) have shed tremendous light on the process of synaptogenesis, though similar studies on synapse formation between central neurons remain in their infancy. One could envisage that the fundamental principles that govern synapse formation at the NMJ may also apply to the CNS neurons, though this assumption has not been supported with experimental data. To cite an example, agrin, which is a key mediator of synapse formation at the NMJ, plays a very limited, if any, role in CNS synapse formation (So et al., 1996; Serpinskaya et al., 1999; Li et al., 1999). While the intact brain's complexity deters further studies on synapse formation, alternative *in vitro* cell culture techniques remain viable options. Indeed, hippocampal neurons in cultures have proven useful for studying synaptic physiology and synapse formation, however, this model remains complex vis-à-vis extensive interactions between neurons, and furthermore, the involvement of glial cells cannot be excluded. Instead, invertebrate neurons from C. *elegans* and *Drosophila* offer simpler brains for studying synapse formation, though neurons from these species are not readily accessible for direct electrophysiological recordings. The mollusks, on the other hand, provide relatively simple nervous systems with neuronal somata and their synapses directly accessible for both morphological and electrophysiological manipulations (Munno and Syed, 2003). For instance, identified neurons from *Helisoma* developed synapses between their somata without neuronal outgrowth, thus providing simultaneous access to somata and synaptic sites (Haydon, 1988). Although these studies have contributed significantly to our understanding of synaptic physiology and its associated morphological changes, the fundamental mechanisms of synapse formation in this species remain elusive. Similarly, in *Aplysia* the same approach was used to reconstruct synapses between identified sensory and motor neurons. The synapses that developed between these neurons were similar to those seen *in vivo* (Klein, 1994), thus validating the usefulness of this model for studies on synaptic physiology and plasticity.
Similarly, identified neurons form *Lymnaea* re-formed mutually inhibitory synaptic connections between the neurites (Syed et al., 1990) as well as their somata (Feng et al., 1997 – for further details see Bulloch and Syed, 1992; Munno and Syed, 2003). The

neurons VD4 and LPeD1 used in this chapter have also been shown to re-form excitatory synapses *in vitro* (Hamakawa et al., 1999) and these synapses were similar to those seen *in vivo*. Moreover, we have demonstrated that these synapses not only re-form in a soma-soma and neurite-neurite configuration, but also in a soma-axon configuration. Furthermore, existing synapses were also maintained in an axon-axon configuration. Taken together with previous studies, the data presented here demonstrate that *Lymnaea* is an excellent model for studying synaptogenesis between the central neurons.

Despite recent progress toward our understanding of the mechanisms underlying synapse formation, so far the precise contributions of pre- and postsynaptic somata in this process remain unclear. Studies over the past several years have led to the idea that reciprocal interactions between pre- and postsynaptic neurons are essential for the assembly of the synaptic machinery that makes future synaptic transmission compatible with the functional needs of the animal. In this chapter, we have shown that the isolated axons can maintain synapses in the absence of their respective somata. Specifically, synapses formed between intact LPeD1 and VD4 were maintained for several days after the removal of their respective somata. These data are consistent with earlier studies on crustacean models which showed that axons severed from their soma *in vivo* survived morphologically, electrophysiologically, and functionally. Specifically, isolated axons generated action potentials, released transmitter, and remained functionally integrated into the host circuit (Atwood et al., 1973; Hoy, 1969; Hoy et al., 1967; Wine, 1973). Similarly, postsynaptic responses to secreted neurotransmitters persisted for a long period of time after the soma removal (Krasne and Lee, 1977). Axonal ability to survive for months, in the absence of its somata, could not be attributed either to the pre-existing housekeeping proteins in the extrasomal compartments or to various mRNA species encoding for these proteins as their half-lives are unlikely to exceed beyond several days. Consistent with this notion are our data whereby synapses between isolated axons of VD4 and LPeD1 were found to run down within 3 days of soma removal. How then did the axotomized neurons from the crayfish survived for several months? A number of interesting scenarios have been proposed. One is the demonstration of protein transfer from surrounding neurons and glial cells to axotomized axons in the squid (Gainer et al., 1977; Lasek et al., 1977) and crayfish (Meyer and Bittner, 1978; Tytell et al., 1986; Sheller and Bittner, 1992). Another possibility may be that somehow the severed axons 'receive' either proteins or their encoding mRNAs from the neighboring axons or glia. Consistent with this notion is the hypothesis of 'glial transfer'. According to this idea, the glial cells in the vicinity of the severed axon 'donate' their nuclei to the injured axons. Indeed, Atwood et al. (1989) found that after its severance from the somata, the injured axons appeared multi-nucleated as if the glial cells had 'donated' their nuclei to the axon (Atwood et al., 1989). Therefore, in the intact animal support cells such as glial and satellite cells, could have accounted for the survival of axotomized axons through 'donating' possibly functional organelles. Because synaptic transmission between VD4 and LPeD1 axons ran down within a couple of days, it is therefore reasonable to assume that in the absence of somata (and also the glia cells) existing synapses may 'run out of fuel' and the incidence of synapse formation is reduced (Figure 3). These data emphasizes the importance of the presynaptic soma in this process and suggest the involvement of genetic programs and protein synthesis for the maintenance of existing synapses.

Previous studies have demonstrated that bidirectional, inductive interactions play important roles in synapse formation. Anterograde signals emanating from growth cone act on the target cells (Goodman and Shatz, 1993; Daniels, 1997), whereas retrograde signals communicate back from targets to influence growth cone behavior (Hall and Sanes, 1993; Haydon and Zoran, 1994; Tao and Poo, 2001). The data in this chapter show that although the presynaptic soma was necessary for synaptogenesis, it is however unclear whether the interactions leading up to synapse formation involved unidirectional or bidirectional communications.

The requirement of the presynaptic soma in synaptogenesis suggests the involvement of gene transcription and protein translation program in this process. In the intact animal, it seems that neurite outgrowth is accompanied by putting the growth cone in "ready-steady-go" state (Haydon and Drapeau, 1995), thus allowing for a rapid synaptogenic program to proceed immediately after the contact between the cells. At the NMJ, it has been demonstrated that evoked synaptic transmission is possible within seconds of nerve-muscle contact (Sun and Poo, 1987). After the muscle cell contact, the presynaptic neurotransmitter release is enhanced by inductive signals from the target cells and this occurs over the time course of several minutes (Xie and Poo, 1986). In addition, it has also been shown that growth cones possess the necessary neurotransmitter release machinery before contact with muscle cells (Young and Poo, 1983; Hume et al., 1983). A recent study showed the presence of YFP-labeled synaptotagmin fusion protein in the cytoplasm, lamellipodia, filopodia and growth cones of outgrowing neurites within 4 hrs in culture (Narayan and Greif, 2004), indicating that the rudimentary synaptic machinery appears concomitant with neurite outgrowth. Although it has been shown that pre- and postsynaptic elements are ready for synaptic transmission prior to contact (Haydon and Drapeau, 1995) and that pre- (Ahmari et al., 2000) and postsynaptic components (O'Brien et al., 1997; Rao et al., 1998; Levi et al., 1999) are assembled prior to target cell contact, in our soma-axon preparation the activation of a genetic program for synaptogenesis seems to be required for new synapse formation. On the other hand, the formation and axonal transport of presynaptic transport vesicles containing the pre-assembled components of the presynaptic vesicle release machinery (Zhai et al., 2001; Shapira et al., 2003; Besler et al., 2004), show consistency with our data in that the formation of these vesicles cannot do without the cell soma. Our data and these studies, again, indicate that at least the presynaptic part of the synapse can only form with the somal contribution.

The dispensability of the postsynaptic soma for synapse formation indicates that axons contain all the required components for the synaptic machinery, either in the form of proteins or various mRNAs encoding for the required proteins. As mentioned earlier, postsynaptic components in neurons have been demonstrated to be ready for synaptogenesis prior to cell contact (Haydon and Drapeau, 1995; O'Brien et al., 1997; Rao et al., 1998; Levi et al., 1999). The importance of postsynaptic neurons in synapse formation has been demonstrated in *Lymnaea* studies by means of *in vitro* antisense knock-down of specific mRNA encoding for multiple endocrine neoplasia type 1 (*MEN1*). The *MEN1* gene encodes for the transcription factor *menin* and is upregulated during synapse formation between VD4 and LPeD1. Postsynaptic antisense knock-down of mRNA encoding this protein blocks the formation of synapses between identified neurons (van Kesteren et al., 2001), indicating that the transcription factor *menin* in the

postsynaptic neuron is important for the development of synapses. Since in our soma-axon model the postsynaptic soma is not available, it is possible that *menin* or its mRNA may have already been transported to the axon prior to its severance from the cell body. In support of this argument are studies whereby isolated axons from *Aplysia* can form new synapses in a protein synthesis dependent manner (Schacher and Wu, 2002). These studies demonstrate that axons are likely equipped with all the required components for synapse formation prior to cell contact. Physical interactions with target neurons could activate the local synthesis of synaptic proteins and their insertion in the plasma membrane.

In summary, the data presented in this chapter validate the usefulness of soma-axon pairs in determining the specificity of synapse formation between identified cells. Moreover, we have demonstrated an innate ability of isolated axons to maintain pre-existing synaptic specializations. Furthermore, these data also show that the presynaptic but not the postsynaptic soma is required for the formation of specific synapses as well as the development of specific synaptic sites between VD4 and LPeD1.

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chapter

3

Synapse Formation Between Soma-Axon Pairs Requires Trophic Factors And Is Mediated Via Receptor Tyrosine Kinases

with Naweed I Syed

Abstract

Synapse formation during development and synaptic plasticity forms the basis for all nervous system functions in the adult animals, though the underlying mechanisms remain largely unexplored. Although the requirements of *de novo* protein synthesis for synapse formation and synaptic plasticity have been documented, unequivocal evidence awaits further studies. In this chapter, we sought to determine the involvement of neuronal protein synthetic machinery and extrinsic trophic factors in synapse formation between isolated *Lymnaea* neurons and their severed axons. The formation of cholinergic synapses between presynaptic soma (VD4) and postsynaptic axon (LPeD1) required gene transcription and protein synthesis solely in the presynaptic neuron. We also show that this synaptogenesis was contingent upon extrinsic trophic factors present in brain conditioned medium (CM). The CM-induced excitatory synapse formation between soma-axon pairs was mediated through receptor tyrosine kinases.

Introduction

In the intact developing brain, neurotrophic factors play many important roles in neuronal development, ranging from cell proliferation, migration, differentiation, survival and neurite outgrowth (Markus et al., 2002; McAllister, 2002; Boyd and Gordon, 2003; Marzella and Gillespie, 2002; Huang and Reichardt, 2001; Yamamoto et al., 2002). Studies on both vertebrates (Vicario-Abejon et al., 1998; Aguado et al., 2003) and invertebrates (De-Miguel, 2000) have confirmed earlier suggestions (Goldowitz and Cotman, 1980) that trophic factors may also regulate synapse formation through their actions on both pre-and postsynaptic neurons. Nerve growth factor (NGF) modulates presynaptic terminals in the adult visual cortex, possibly by inducing the formation of new synapses (Liu et al., 1996). Similarly, BDNF and NT-3 were found to increase the total number and the number of docked synaptic vesicles at the presynaptic terminals (Collin et al., 2001). BDNF and NT-4 inhibited agrin-induced clustering of AChRs on cultured myotubes, suggesting an interplay between agrin and neurotrophic factors in regulating the formation of postsynaptic specializations (Wells et al., 1999 – see Vicario-Abejon et al., 2002 for further details). Whether trophic factors affect excitatory or inhibitory synapse formation differentially and whether these actions involve gene transcription and *de novo* protein synthesis remains unknown. In *Lymnaea*, trophic factors were found to be important for excitatory but not the inhibitory synapse formation (Hamakawa et al., 1999; Munno et al., 2000). Moreover, it was demonstrated that in the absence of trophic factors, neurons established inappropriate inhibitory synapses, which could, however be corrected by the addition of appropriate trophic molecules present in the CM. Taken together, these studies show the requirement of trophic factors in synapse formation in both vertebrate and invertebrates, though the underlying mechanisms remain unknown.

Recent studies on isolated axons from cultured *Aplysia* neurons demonstrate the requirement of *de novo* protein synthesis in the formation and modulation of newly formed synaptic connections, though the precise site (i.e. pre verses postsynaptic) for this protein synthesis and the underlying mechanisms remain unresolved. For instance,

Trudeau and Castellucci (1995), and Martin et al. (1997), have shown that long-term synaptic potentiation (which requires new synapses), at the sensorimotor synapse does not involve new protein synthesis in the postsynaptic cell (motor neuron), whereas Sherff and Carew (1999) have shown that blocking protein synthetic machinery in postsynaptic neurons prevents long-term facilitation. Coulson and Klein (1997) on the other hand, showed that neither pre- nor postsynaptic protein synthesis is required for synapse formation and synaptic plasticity at soma-soma synapses between cultured *Aplysia* neurons. In contrast, Feng et al. (1997), have shown that synaptogenesis between paired *Lymnaea* somata is contingent upon *de novo* protein synthesis. More recently, Schacher and Wu (2002) have shown that, although protein synthesis in both pre- and postsynaptic axons is required for the maintenance of pre-existing synapses, these steps did not, however, involve the soma of either cell.

In this chapter, we show that the formation of cholinergic synapses between presynaptic soma and postsynaptic axon pairs requires gene transcription and protein synthesis specifically in the presynaptic neuron. Moreover, this synaptogenesis is contingent upon extrinsic trophic factors and is mediated through receptor tyrosine kinases. However, neither protein synthesis nor gene transcription is required postsynaptically for synapse formation. This chapter thus provides evidence that trophic factors exert unique actions on both pre- (protein synthesis and gene transcription) and postsynaptic neurons (protein synthesis independent) to bring about specific changes that are essential for synapse formation.

Materials and Methods

Animals. Lymnaea stagnalis were maintained at room temperature in a well-aerated aquarium containing filtered water. For experiments involving cell isolation, snails approximately 1-2 months old (shell length 18-20 mm) were used, while conditioned medium (CM) was prepared from 2-3 month old animals (shell length 25-30 mm).

Cell Culture. Neurons were isolated from the central ring ganglia and maintained in cell culture as described previously (Syed et al., 1990; Ridgway et al., 1991; Syed et al., 1999). Briefly, snails were anesthetized with 10% Listerine solution (ethanol, 21.9%; methanol, 0.042%) in normal *Lymnaea* saline [(in mM): 51.3 NaCl, 1.7 KCl, 4.0 $CaCl_2$ and 1.5 $MgCl_2$] buffered to pH 7.9 with HEPES. The central ring ganglia were then washed several times (3 washes, 15 min each) with normal saline containing antibiotic (gentamycin, 50 μg/ml). The central ring ganglia were then treated with enzyme (trypsin) followed by enzyme inhibitor (trypsin inhibitor) and pinned down at the bottom of a dissection dish. All procedures were performed under sterile culture conditions.

Conditioned medium (CM) was prepared by incubating gentamycin (20 μg/ml)-treated ganglia in Sigmacote-treated glass petri dishes, containing defined medium (DM, L-15; Life Technologies, Gaithersburg, MD; Special Order). DM consisted of serum free, 50% L-15 medium with added inorganic salts (in mM: 40 NaCl, 1.7 KCl, 4.1 $CaCl_2$, 1.5 $MgCl_2$, and 10 HEPES, pH 7.9) and 20 μM gentamycin. The ganglia were incubated in a

humidifier for 3 - 4 days (Syed et al., 1999; Wong et al., 1981) and the resulting CM was frozen (-20^{o} C) until used.

The identified neurons were isolated by applying gentle suction through a fire-polished, Sigmacote (Sigma, St. Louis, Mo.)-treated pipette. The isolated neurons were then plated on poly-L-lysine-pretreated glass coverslips (Ridgway et al., 1991) in either DM or CM. Axons were isolated by first plating the cell body along with its intact axon segment in cell culture and allowing it to adhere to the poly L-lysine coated dish. The axon was then immediately severed from the cell body by using a sharp glass pipette, and the severed cell body was subsequently removed from the culture dish. Soma-axon synapses were prepared juxtaposing the soma to the isolated axon. In some experiments, isolated cells were initially plated on hemolymph-pretreated culture dishes (to prevent adhesion) containing CM. After 12-18 hours, the cells were transferred to normal poly L-lysine coated dishes and paired in CM.

For experiments involving anisomycin pretreatment, LPeD1 axon was cultured on poly L-lysine coated dishes containing CM alone or CM + anisomycin. After 12-18 hours, the CM containing anisomycin was replaced with fresh CM and VD4 was paired with the axon. The VD4 was first maintained in hemolymph pre-treated dishes containing either CM alone or CM + anisomycin. After 12 - 18 hours, VD4 was removed from hemolymph pre-treated dishes and paired with LPeD1 axon on normal poly L-lysine dishes containing CM.

Electrophysiology. Neuronal activity was monitored using conventional intracellular recording techniques, as described previously (Syed and Winlow, 1991). Glass microelectrodes (1.5 μm internal diameter; World Precision Instruments, Sarasota, FL) were filled with a saturated solution of K_2SO_4 (resistance, 20-40 MΩ). An inverted microscope (Axiovert 135; Zeiss, Thornwood, NY) was used to view the neurons, which were impaled using Narashige (Tokyo, Japan) micromanipulators (MM202 and MM 204). Amplified electrical signals (Neuro Data Instrument Corp.) were displayed on a digital storage oscilloscope (PM 3394; Philips, Eindhoven, The Netherlands) and recorded on a chart recorder (TA 240S; Gould, Cleveland, OH).

Transcription, translation and receptor tyrosine kinase experiments. To test whether synapse formation was mediated through receptor tyrosine kinases (RTK), a non-specific, RTK blocker Lavendustin A (LavA, 10 μM), and its inactive analog, Lavendustin B (LavB, 10 μM) were used. Gene transcription and protein synthesis were perturbed by actinomycin D (1 μg/ml) and anisomycin (12.5 μg/ml) respectively.

Chemicals. Mecamylamine, anisomycin, actinomycin-D, Lavendustin A and Lavendustin B were obtained from Sigma. *Lymnaea*-EGF was extracted and purified from *Lymnaea* albumen glands by Gregg T. Nagle, PhD, University of Texas Med Br, Marine Biomed Inst, Med Res Bldg, Galveston.

Results

The synaptic transmission between VD4-soma and LPeD1-axon is cholinergic.
Excitatory synapses between the specific soma-soma paired *Lymnaea* neurons VD4 and LPeD1 are cholinergic (Woodin et al., 2002), although the VD4 has been shown previously to contain and release a variety of peptides *in vivo* (Skingsley et al., 1993). To determine the nature of synaptic transmission between VD4-soma and LPeD1-axon, soma-axon pairs were cultured and synapses tested. Subsequently, these pairs were exposed to the ACh antagonist mecamylamine (1 μm). The VD4-induced EPSPs in LPeD1 were blocked completely and reversibly (Figure 1) by this antagonist (n=6), suggesting that, as observed *in vivo* and also in the soma-soma configuration (Woodin et al., 2002), the synaptic transmission between VD4-soma and LPeD1-axon is cholinergic as well.

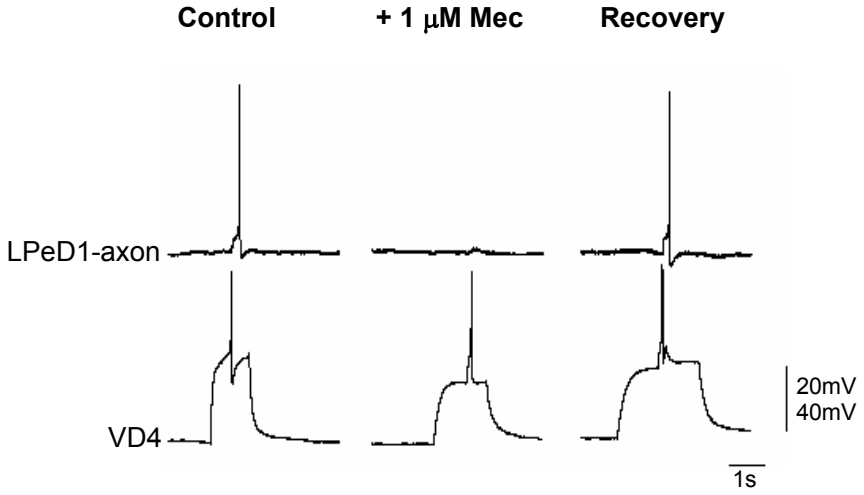

Figure 1: Soma-axon synapse is cholinergic.
To demonstrate the chemical and cholinergic nature of synaptic transmission between VD4-soma and LPeD1-axon, synapses were tested either in the presence or absence of ACh antagonist. Mecamylamine (1 μM) completely and reversibly blocked synaptic transmission between VD4-soma and LPeD1-axon, suggesting that like soma-soma pairs (Woodin et al., 2002), this synaptic transmission is cholinergic (n=6).

Excitatory synapse formation between VD4-soma and LPeD1-axon pairs requires extrinsic trophic factors.
Excitatory synapse formation between soma-soma paired *Lymnaea* neurons requires trophic factor mediated activation of receptor tyrosine kinases, which in turn induces synapse specific gene transcription and *de novo* protein synthesis (Hamakawa et al., 1999). To test whether soma-axon synaptogenesis also requires extrinsic trophic factors, soma-axon pairs were cultured in the presence (CM) or absence (DM) of trophic factors. Intracellular recordings revealed that when paired in CM, 100% of VD4-soma and LPeD1-axon pairs developed excitatory synapses (n=50) (Figure 2A), whereas in DM 88% of the pairs failed to develop synapses (n=25) (Figure 2B). A summary of soma-

axon synapse formation in CM and DM is presented in Figure 2C. It is important to note that the neurons comprising the soma-axon pairs exhibited electrophysiological characteristics in DM that were indistinguishable from the characteristics seen in CM (See chapter 4 for details). These experiments thus demonstrate the requirement of CM-derived trophic factors in synapse formation between soma-axon pairs.

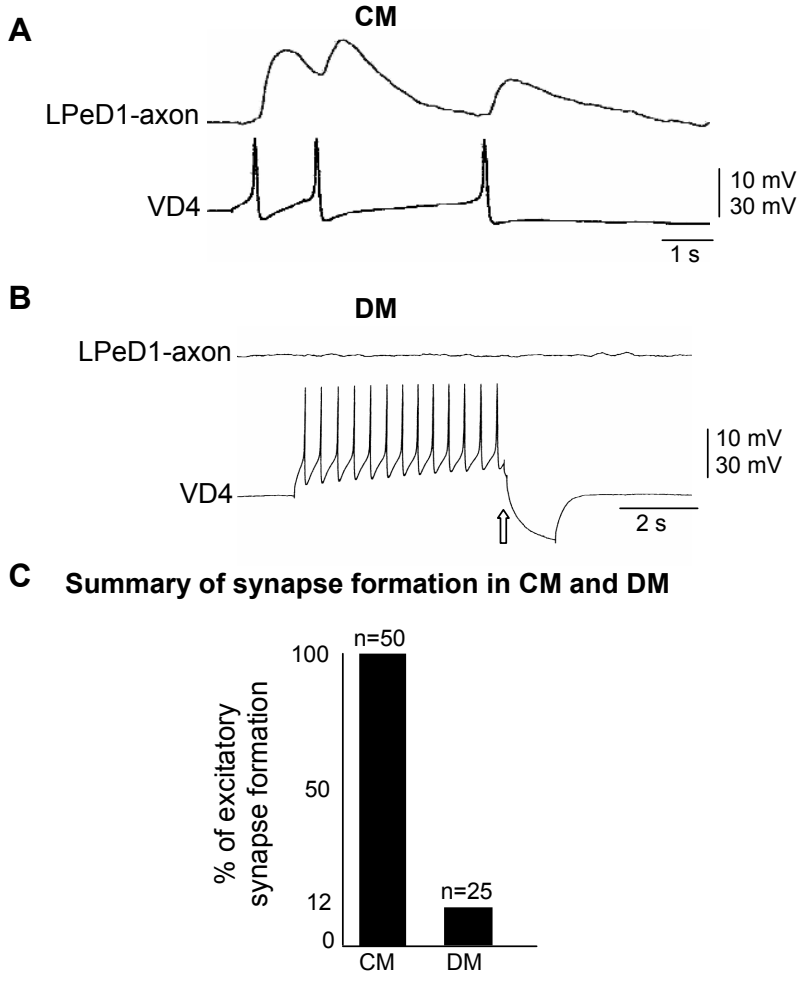

Figure 2: Soma-axon synapse formation requires trophic factors.
To test the requirement of trophic factors for excitatory synapse formation between VD4-soma and LPeD1-axon, soma-axon pairs were cultured in DM or in CM. (**A**) When paired in CM, excitatory synapses were detected between all soma-axon pairs (n=50), whereas in (**B**) DM 88% of the soma-axon pairs failed to develop excitatory synapses (n=25). Specifically, (**A**) induced action potentials in the VD4 paired with the LPeD1-axon in CM generated 1:1 EPSPs. (**B**) Similar pairing in DM and subsequent VD4 stimulation did not reveal excitatory synapses between the pairs. Note that hyperpolarizing current injection in the VD4 (at open arrow) also did not reveal the presence of electrical coupling between the pairs. A summary of soma-axon synapse formation in CM and DM is presented in (**C**).

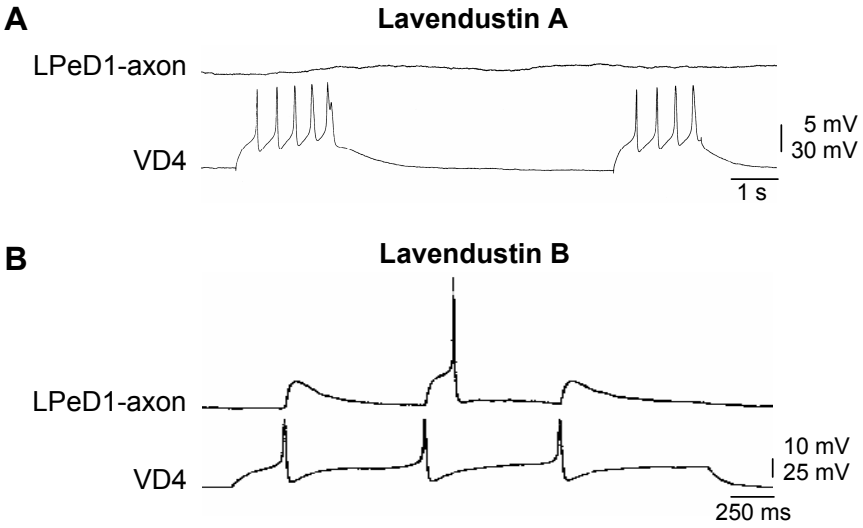

Figure 3: Soma-axon synapse formation is mediated by receptor tyrosine kinases.
To test whether CM-induced synapse formation was mediated by receptor tyrosine kinases, soma-axon pairs were cultured either in the presence of the receptor tyrosine kinase inhibitor Lavendustin A (10 μM) (n=8) or its inactive analogue Lavendustin B (10 μM) (n=8). (**A**) Pairs cultured in the presence of LavA failed to develop excitatory synapses, (**B**) whereas normal synapses developed in LavB.

Excitatory synapse formation between VD4-soma and LPeD1-axon pairs is mediated by Receptor Tyrosine Kinases.

Because trophic factor-induced excitatory synapse formation is known to involve receptor tyrosine kinase (RTK) activity, we next tested whether synapse formation between soma-axon pairs also requires CM mediated activation of RTK. VD4-soma and LPeD1-axon were paired in CM containing either Lavendustin A (LavA, a RTK inhibitor, 10 μM) or its inactive isoform Lavendustin B (LavB, 10 μM). LavA (Figure 3A, n=8), but not LavB (Figure 3B, n=8) completely blocked excitatory synapse formation between VD4-soma and LPeD1-axon, demonstrating that the CM-induced excitatory synapse formation is mediated via RTK activity. Although the precise identity of the synapse specific trophic molecules in CM is presently unknown, *Lymnaea* Epidermal Growth factor (L-EGF) has previously been shown to mimic the CM-induced effects on neurite outgrowth (Hermann et al., 2000) and synapse formation (Hamakawa et al., 1999) between soma-soma paired *Lymnaea* neurons. To test whether L-EGF (which induces gene transcription and protein synthesis through RTK activation (Cohen et al., 1981; Hunter and Cooper, 1981; Leutz and Schachner, 1981; Murdoch et al., 1982)) could substitute for CM vis-à-vis synaptogenesis, we cultured soma-axon pairs in DM + L-EGF (100 nM). After 12-18 hours, excitatory synapses were detected between 6 of these pairs (n=7), suggesting that L-EGF can mimic the CM-induced effects on excitatory synapse formation (Figure 4).

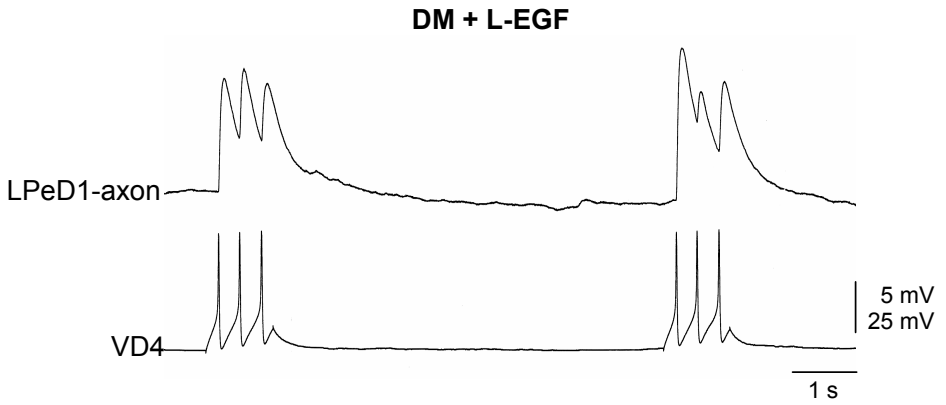

Figure 4: CM-induced excitatory synapse formation is mimicked by L-EGF.
To test whether the CM-induced effects could be mimicked by EGF, which is known to act through receptor tyrosine kinases, soma-axon pairs were cultured in DM + 100 nM *Lymnaea*-EGF (L-EGF). The formaton of excitatory synapses was promoted in 86% of the soma-axon pairs (n=7). Specifically, action potentials in the VD4 induced 1:1 EPSPs in the LPeD1-axon, indicating that L-EGF mimics the CM-induced effects on excitatory synapse formation.

Excitatory synapse formation between VD4-soma and LPeD1-axon pairs requires *de novo* protein synthesis and gene transcription.

Next, to determine whether the CM-induced excitatory synapse formation involved *de novo* protein synthesis, VD4 soma and LPeD1 axon were paired in CM containing the protein synthesis blocker anisomycin (12.5 µg/ml). Anisomycin (n=6) completely blocked synapse formation between VD4-soma and LPeD1-axon, suggesting that *de novo* protein synthesis is required for synapse formation between soma-axon pairs (Figure 5A). To determine whether the CM-induced excitatory synapse formation involved gene transcription, VD4 soma and LPeD1 axon were paired in CM containing the transcription inhibitor (actinomycin-D, 1 µg/ml). Actinomycin-D (n=6) also completely blocked synapse formation between VD4 soma and LPeD1 axon, suggesting that gene transcription is required for synapse formation between soma-axon pairs (Figure 5B). To summarize, we have demonstrated that excitatory synapse formation between VD4-soma and LPeD1-axon requires trophic factor-induced gene transcription and *de novo* protein synthesis, which is mediated through RTKs (Figure 6). This is consistent with previous studies, which show that excitatory synaptogenesis between paired somata requires trophic factor-induced gene transcription and *de novo* protein synthesis, which is mediated through RTKs (Woodin et al., 1999; Hamakawa et al., 1999). These data thus demonstrate that the cellular mechanisms underlying synapse formation between soma-soma and soma-axon pairs follow a common pathway. Although the trophic factor-induced gene transcription involves the presynaptic soma, these data do not identify the precise site (i.e. presynaptic versus postsynaptic) at which the trophic factor-induced protein synthesis occurs.

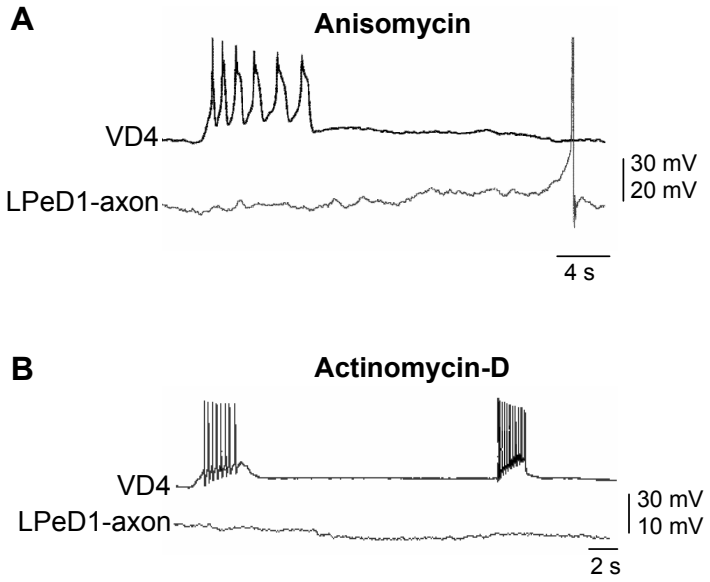

Figure 5: Soma-axon synapse formation requires *de novo* protein synthesis and gene transcription.
(**A**) To test the requirement of protein synthesis for soma-axon synapse formation, VD4-soma and LPeD1-axon were paired in the presence of the protein synthesis inhibitor anisomycin (12.5 μg/ml) (n=6). Under these circumstances soma-axon pairs failed to develop excitatory synapses. (**B**) In order to test whether synapse formation between VD4-soma and LPeD1-axon required gene transcription, these soma-axon pairs were cultured in the presence of the gene transcription inhibitor actinomycin-D (1μg/ml) (n=6). Pairs cultured under these circumstances did not develop excitatory synapses.

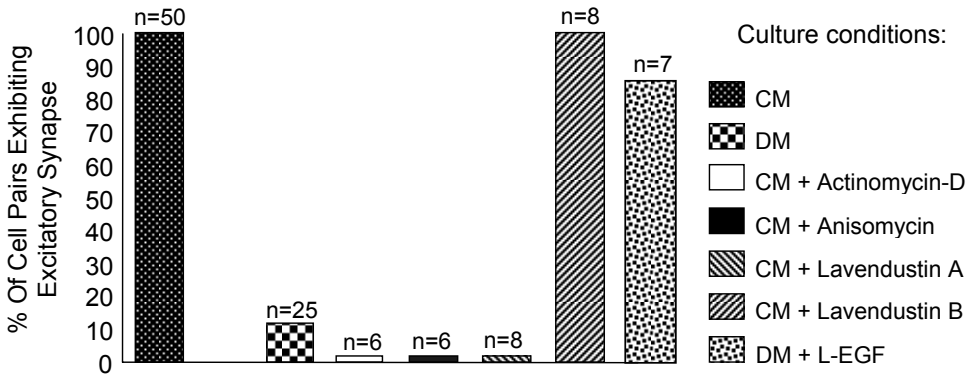

Figure 6: Summary data depicting the incidence of soma-axon synapse formation.
The formation of excitatory synapses between soma-axon pairs has been shown to require trophic factors. CM-induced gene transcription and *de novo* protein synthesis have also been shown to be important for the development of soma-axon synapses. Further, CM-induced effects are mediated by receptor tyrosine kinases and are mimicked by L-EGF.

Does the protein synthesis dependent step underlying excitatory synapse formation involve the presynaptic soma or the postsynaptic axon?

To test whether the CM-induced protein synthesis-dependent step underlying synapse formation occurred in the presynaptic soma or the postsynaptic axon, both were pretreated separately with anisomycin (12.5 μg /ml) for 12-24 hours in CM. When both the soma and the axon were directly paired in CM + anisomycin, and synaptic connections were tested under normal recording conditions, no synapses were detected between the paired cells (see Figure 5A). Next, either VD4-soma or LPeD1-axon were independently pre-treated with anisomycin overnight (see methods), and subsequently paired in CM for 5 hours prior to intracellular recordings. We found that blocking presynaptic (n=7, Figure 7A), but not postsynaptic (n=6, Figure 7B) protein synthesis prior to pairing, perturbed synapse formation. These data, summarized in Figure 8, show that postsynaptic axon does not require *de novo* protein synthesis for synapse formation and that the protein synthesis dependent step underlying synaptogenesis occurs only in presynaptic somata.

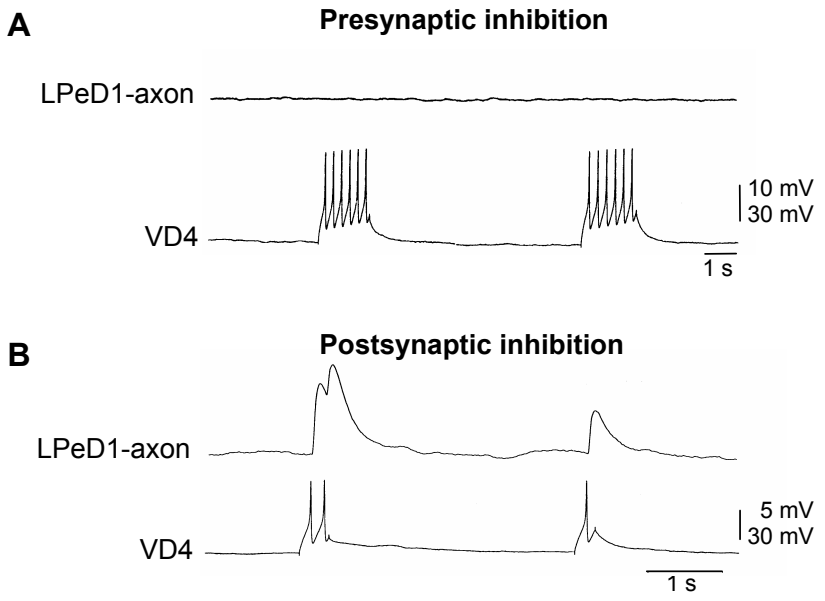

Figure 7: Presynaptic soma, but not postsynaptic axon, requires protein synthesis for excitatory synapse formation.

(**A**) Pretreatment of VD4-soma alone with the protein synthesis inhibitor anisomycin, prior to its pairing with LPeD1-axon, prevented synapse formation between the identified neurons (n=7). Induced action potentials in the VD4 failed to generate EPSPs in the LPeD1-axon. (**B**) Pretreatment of LPeD1-axon alone with anisomycin, prior to pairing with VD4-soma, did not affect synapse formation between these pairs (n=6). Specifically, intracellular recordings revealed that action potentials induced in VD4s paired with anisomycin pre-treated LPeD1-axons generated 1:1 EPSPs that were indistinguishable from those seen under control conditions.

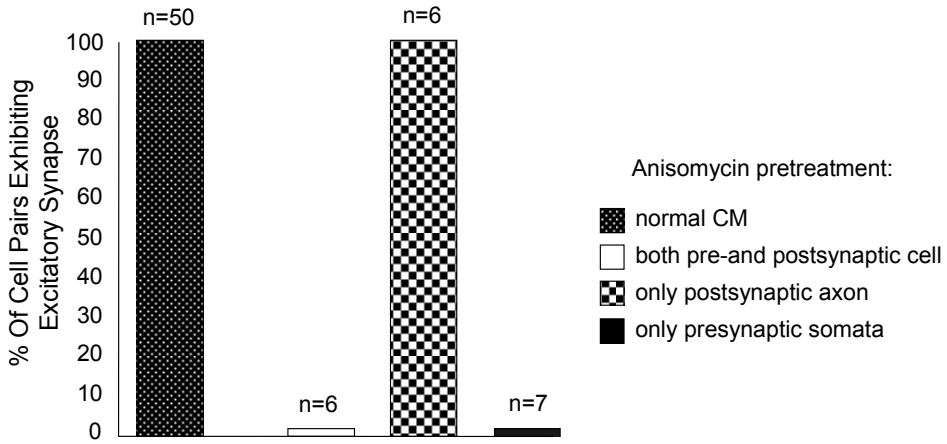

Figure 8: Summary data showing the requirement of protein synthesis for synapse formation.
In CM, VD4 soma and LPeD1 axon established excitatory synapse. The CM-induced excitatory synapse formation was blocked when soma-axon pairs were maintained in CM containing anisomycin (n=6). Note that these data are identical to those presented in Figure 6, and are used here only for comparative purposes. Pretreatment of the LPeD1-axon alone, prior to pairing, had no effect on synapse formation, whereas pairs of which the VD4-soma was pretreatment prior to pairing failed to develop any detectable excitatory synapses.

Discussion

The results described in this chapter demonstrate that cell-cell signaling and extrinsic trophic factors act in concert to bring about specific changes in both pre- and postsynaptic partners during synaptogenesis. In the case of the presynaptic neuron, these changes invoke both the genetic and protein synthetic machinery, whereas the postsynaptic partner does not require gene transcription or *de novo* protein synthesis.

Both pre-and postsynaptic neurons are deemed ready for synapse formation prior to contact with their synaptic partners (Haydon and Drapeau, 1995). However, cell-cell signaling, which often requires new protein synthesis, plays a pivotal role in the maturation and consolidation of newly formed synapses. Indeed Martin et al. (1997) have shown that the precise site for the synthesis of synapse/plasticity specific proteins is the presynaptic cell. Consistent with these studies are our data, which have shown that the presynaptic but not the postsynaptic soma is required for new synapse formation. Furthermore, as shown earlier, although the isolated *Lymnaea* axons are capable of protein synthesis (van Minnen et al., 1997; van Minnen and Syed, 2001), new proteins are however not required in the postsynaptic LPeD1-axon for synapse formation. Because treatment of the presynaptic cell with either transcription or translation inhibitors blocked synapse formation, our data support the hypothesis that both transcription and translation machinery required for excitatory synapse formation between *Lymnaea* neurons involves the presynaptic cell.

Although the requirement of presynaptic protein synthesis for synapse formation in *Lymnaea* is consistent with similar roles in synaptic plasticity in *Aplysia*, these studies differ from those of Schacher and Wu (2002), who have shown that neither presynaptic nor postsynaptic soma is required for new synapse formation. A potential explanation for the discrepancy between these studies may be that in our experiments, axons were severed from their soma immediately after neuronal extraction, whereas Schacher and Wu (2002) allowed axons to grow first for at least two days before their somata were removed. It is therefore plausible that the isolated *Aplysia* axons may have had the opportunity to transport and harbor various mRNA and their encoded proteins prior to soma removal, especially given the findings of transport of presynaptic components to the axon terminal in vertebrate neurons. Thus, these transported molecules may have subsequently been used to facilitate synaptic transmission in the *Aplysia* model. Recent studies now show that local protein synthesis is not only essential for new synapse formation in some models, but that this process is also involved in regulating the plasticity of existing synapses. In *Aplysia* for instance, long term, albeit transient facilitation (LTF), has been shown to be dependent on local protein synthesis (Liu et al., 2003). Similarly, in hippocampal neurons long-term potentiation (LTP) at Schaffer-commissural synapses on pyramidal cells also relies upon local protein synthesis (Bradshaw et al., 2003). In support of this local protein synthesis dependent LTP is the evidence showing that dendritic spines containing a larger number of polysibosomes appear after tetanic stimulation. The presence of polyribosomes in these spines suggests a protein synthesis dependent stabilization of the enlarged postsynaptic density (Ostroff et al., 2002). All these studies show the importance of local protein synthesis in LTF and LTP at mature synapses. Only a few studies (Schacher and Wu, 2002; Martin et al., 1997) however, demonstrate the involvement of local protein synthesis in new synapse formation. From the above studies, it therefore appears that somata based transcription and translation is critical during initial stages of synapse formation, synaptic plasticity and synapse maintenance, whereas subsequent short-term morphological and functional changes may invoke local protein synthesis (Martin et al., 1997). This notion is consistent with data presented in this study where gene translation and protein synthesis in the presynaptic cell body was required for new synapse formation, whereas neither the presynaptic nor the postsynaptic somata were essential for the short-term (24-48hrs) maintenance of synapses between VD4 and LPeD1. However, as the synaptic strength between axon-axon pairs began to decline over the course of time, our data also demonstrate the essential role of soma-based transcription and translation in the maintenance of synaptic transmission (see Chapter 2). It is however, unclear whether this decline in synaptic strength involved the run down of the structural (channels, PSD-95, actin, tubulin etc.) or functional proteins (transmitter vesicles, receptors etc).

Although the soma-axon synapse shows similarities with the soma-soma synapse, some interesting differences exist. For instance, in the absence of trophic factors, soma-soma pairs establish reciprocal, inhibitory synapses, which do not normally exist *in vivo* and are hence referred to as "inappropriate". When supplied with trophic factors, these inappropriate synapses disappear and appropriate excitatory synapses are re-formed (Woodin et al., 1999). The soma-axon situation did not however emulate the soma-soma scenario vis-à-vis the DM-induced formation of inappropriate inhibitory synapse. Specifically, in the absence of trophic factors the soma-axon pair did not develop the

anticipated, inappropriate inhibitory synapses. One possibility raised is that either both pre-and postsynaptic somata are required for the formation of inappropriate inhibitory synapse or that the receptors mediating inhibitory responses in the soma are absent on the axon. Consistent with this idea are the data where exogenously applied ACh failed to elicit inhibitory responses in isolated LPeD1-axons maintained in DM (see Chapter 4), suggesting that the axonal inability to form inappropriate inhibitory synapses may be due to the fact that the cholinergic, inhibitory receptor are only located at the soma (see Chapter 4). Similarly, we also noted in some instances that the soma-soma pairs exhibited electrical coupling, which was never the case for soma-axon pairs. These interesting observations although not investigated thoroughly here, do nevertheless suggest the requirement of both somata for the formation of gap junctions. Specifically, it appears that both neurons may have to synthesize proteins called connexins and that these proteins cannot be synthesized or inserted *de novo*, in the absence of either somata. Future studies can employ this model to investigate the cellular and molecular mechanisms by which gap-junctional proteins are synthesized and inserted in the extraxomal compartments.

The data presented here show that although gene transcription and the protein synthesis dependent steps are important for synapse formation between *Lymnaea* neurons, these proteins are neither transcribed nor inserted in the absence of trophic factors. The requirement of trophic factors for synapse formation has also been demonstrated in embryonic hippocampal neuronal cultures. Treatment of rat hippocampal neurons with BDNF and NT-3 is essential for the formation of fast synaptic connections (Vicario-Abejon et al., 1998) and synaptic transmission (Collin et al., 2001). Overexpression of BDNF in hippocampal slices has been shown to increase the number of synapses, most of which are GABAergic (Aguado et al., 2003). Although the expression of GABA and glutamate ionotropic receptors was not altered by BDNF, it did nevertheless mediate conversion of GABA responses from depolarizing to hyperpolarizing through K(+)/Cl(-) co-transporter controlled Cl(-) potentials (Aguado et al., 2003). Another approach to determine the involvement of BDNF in synapse formation was to tag the synaptic marker synaptobrevin II with green fluorescent protein (GFP), which revealed that exposure of *Xenopus* optic neurons to this trophic factor increased synapse numbers at axonal terminals (Alsina et al., 2001). The presence of another synaptic marker, SNAP-25, has also been shown in BDNF secreting cells, suggesting BDNF's involvement in synaptogenesis (Yee et al., 2003). Consistent with these studies are experiments on *Lymnaea* soma-soma models showing that trophic factors are also required for excitatory synapse formation between *Lymnaea* neurons (Hamakawa et al., 1999) and their isolated axons (this chapter). In addition to central synapses, the involvement of receptor tyrosine kinases in synapse formation has been demonstrated extensively at the NMJ. Activation of the tyrosine kinase MuSK is essential for the development of postsynaptic membranes at the NMJ (Jones et al., 1999; DeChiara et al., 1996).

Other receptor tyrosine kinases such as Eph receptors have also been shown to be important for hippocampal dendritic spine formation and for synaptic formation and maturation (Henkemeyer et al., 2003). Furthermore, direct interactions between receptor tyrosine kinases and synaptic receptors have been demonstrated (Dalva et al., 2000). EphB receptors were found to interact with NMDA receptors, suggesting that this

interaction and EphB receptor activation are critical for synaptic development. Similarly, activation of receptor tyrosine kinase and neurotrophin receptor TrkB in cerebellum neurons has been shown to be essential for the development of GABAergic synapses (Rico et al., 2002). Moreover, soma-soma synapse formation between identified *Lymnaea* neurons has been shown to require receptor tyrosine kinase activation (Hamakawa et al., 1999). Although the precise identity of specific trophic molecules present in CM has not yet been determined, EGF-like molecules have been shown to mimic the synaptogenic role of the conditioned medium. For instance, Hamakawa et al. (1999), and the results presented in this chapter, have demonstrated that the CM-induced effects on excitatory synapse formation are mimicked by *Lymnaea* EGF (L-EGF),

Collectively, the data presented in this chapter underscore the importance of trophic factors in the formation of new synapses and show that soma-axon synapse formation is dependent on trophic factor-induced gene transcription and *de novo* protein synthesis which is mediated by receptor tyrosine kinases. These data also suggest that trophic factor mediated events may act in parallel with other transcription and translation dependent mechanisms to determine synapse specificity in the formation of neuronal networks.

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chapter

4

On The Role Of The Isolated Axon

with Nina Van, David W Munno, Jan van Minnen and Naweed I Syed

Abstract

In order to determine the role of isolated LPeD1-axons in the formation of synapses, we investigated the effects of trophic factors and protein synthesis on growth, neurotransmitter receptor expression and other intrinsic membrane properties of single isolated axons. In this chapter we demonstrate that trophic factors induce neurite outgrowth from isolated axons. However, this outgrowth does not depend on protein synthesis, although components of the synthesis machinery are present in the axons. Furthermore, we determined the effects of trophic factors and protein synthesis on the axon's neurotransmitter receptor expression and its intrinsic membrane properties. We show that isolated LPeD1-axons maintain their expression of neurotransmitter receptors, in contrast to LPeD1 somata, and their intrinsic membrane properties in the absence of trophic factors and protein synthesis, with the exception of action potential amplitudes, which demonstrated to be affected by inhibition of the synthesis of proteins.

Introduction

Trophic factors play many important roles in a variety of developmental processes (Davies, 1994; Markus et al., 2002; McAllister, 2002; Boyd and Gordon, 2003; Marzella and Gillespie, 2002; Huang and Reichardt, 2001; Yamamoto et al., 2002) including neuronal sprouting and synaptic plasticity in the adult brain (Black, 1999; Woodin et al., 1999; Levine and Black, 1997).

The involvement of trophic factors in neuronal sprouting has been demonstrated for a variety of neurons. BDNF (Mamounas et al., 2000), NT-3 (Li and Bernd, 1999; Saffrey et al., 2000) and NT-4 (Bosco and Linden, 1999) all have been shown to promote neuronal growth. In addition to their effects on neurite outgrowth and sprouting, trophic factors have also been shown to exert effects on the expression of neurotransmitters, their receptors, and ion channels, thereby regulating neuronal excitability. The actions of neurotrophins have been demonstrated to up-regulate (Eva et al., 1992; Bulleit and Hsieh, 2000) or down-regulate (Eva et al., 1992; Brandoli et al., 1998) the expression of neurotransmitter receptors. Similarly, the functional expression of a variety of currents, such as K^+ (Martin-Caraballo and Dryer, 2002), Na^+ and Ca^{2+} (Grumolato et al., 2003; Vidaltamayo et al., 2002) currents, are also regulated by neurotrophins. These studies demonstrate that neurotrophic factors can exert a variety of effects on ion channels and neurotransmitter receptor expression, which in turn may alter neuronal excitability in the nervous system.

Strong indications for local requirement of neurotrophins have come from studies using compartmentalized neuronal cultures. Culture dishes divided into three chambers allowed Campenot's lab to selectively expose different parts of neurons to a variety of different neurotrophins simultaneously. By culturing adult rat dorsal root ganglion (DRG) neurons in these three-chambered dishes, these researchers have demonstrated that not a single neurotrophin was required for the proximal neurite outgrowth of neurons. However, NGF was required for the distal neurite outgrowth and regeneration of the same neurons (Campenot, 1994; Kimpinski et al., 1997), indicating that neurite growth is a local response to focally applied NGF. These studies did not rule out the possibility that

trophic factors may have been internalized and subsequently transported to the soma inducing gene transcription and protein synthesis. They merely demonstrate that Trk receptors are present on the neurites and are there activated by NGF, which is followed by neurite outgrowth. TrkA (NGF receptor) activation has since been shown to be important in axons, but not in cell bodies, for local growth and retrograde survival signaling (MacInnis et al., 2003). Taken together, it is clear from the above-cited examples that neurotrophins influence a variety of cellular processes and evoke various neuronal responses. However, the exact cellular changes that neurotrophic factors evoke locally on distal compartments have not been fully defined.

In this chapter, we show that trophic factors do act locally on isolated axons by inducing sprouting that does not occur in the absence of trophic factors. We investigated whether these effects are mediated by local *de novo* protein synthesis. Although components of the protein synthetic machinery are present in the axon, sprouting does not depend on protein synthesis. In addition to effects on sprouting, the synthesis of proteins *de novo* in isolated axons is also not required for the expression of AChRs or ion channels to maintain the axons excitability, resting membrane potential and spiking threshold. However, we found the amplitude of action potentials to be affected by inhibition of protein synthesis, suggesting that local protein synthesis, possibly induced by trophic factors, does occur in the axon and seems to serve more of a long-term role in the maintenance of excitability.

Materials and Methods

Animals. Lymnaea stagnalis were maintained at room temperature in a well-aerated aquarium containing filtered water. For experiments involving cell isolation, snails approximately 1-2 months old (shell length 18-20 mm) were used, while conditioned medium (CM) was prepared from 2-3 month old animals (shell length 25-30 mm).

Cell Culture. Neurons were isolated from the central ring ganglia and maintained in cell culture as described previously (Syed et al., 1990; Ridgway et al., 1991; Syed et al., 1999). Briefly, snails were anesthetized with 10% Listerine solution (ethanol, 21.9%; methanol, 0.042%) in normal *Lymnaea* saline [(in mM): 51.3 NaCl, 1.7 KCl, 4.0 $CaCl_2$ and 1.5 $MgCl_2$] buffered to pH 7.9 with HEPES. The central ring ganglia were then washed several times (3 washes, 15 min each) with normal saline containing antibiotic (gentamycin, 50 μg/ml). The central ring ganglia were then treated with enzyme (trypsin) followed by enzyme inhibitor (trypsin inhibitor) and pinned down at the bottom of a dissection dish. All procedures were performed under sterile culture conditions.

Conditioned medium (CM) was prepared by incubating gentamycin (20 μg/ml)-treated ganglia in Sigmacote-treated glass petri dishes, containing defined medium (DM, L-15; Life Technologies, Gaithersburg, MD; Special Order). DM consisted of serum free, 50% L-15 medium with added inorganic salts (in mM: 40 NaCl, 1.7 KCl, 4.1 $CaCl_2$, 1.5 $MgCl_2$, and 10 HEPES, pH 7.9) and 20 μM gentamycin. The ganglia were incubated in a humidifier for 3 - 4 days (Syed et al., 1999; Wong et al., 1981) and the resulting CM was frozen (-20^{o} C) until used.

The identified neurons (somata and initial axon segment) were isolated by applying gentle suction through a fire-polished, Sigmacote (Sigma, St. Louis, Mo.)-treated pipette. The isolated neurons were then plated on poly-L-lysine-pretreated glass coverslips (Ridgway et al., 1991) in either DM or CM. Axons were isolated by first plating the cell body along with its intact axon segment in cell culture and allowing it to adhere to the poly L-lysine coated dish. The axon was then immediately severed from the cell body by using a sharp glass pipette, and the cell body was subsequently removed from the culture dish.

Electrophysiology. Neuronal activity was monitored using conventional intracellular recording techniques, as described previously (Syed and Winlow, 1991). Glass microelectrodes (1.5 μm internal diameter; World Precision Instruments, Sarasota, FL) were filled with a saturated solution of K_2SO_4 (resistance, 20-40 MΩ). An inverted microscope (Axiovert 135; Zeiss, Thornwood, NY) was used to view the neurons, which were impaled using Narashige (Tokyo, Japan) micromanipulators (MM202 and MM 204). Amplified electrical signals (Neuro Data Instrument Corp.) were displayed on a digital storage oscilloscope (PM 3394; Philips, Eindhoven, The Netherlands) and recorded on a chart recorder (TA 240S; Gould, Cleveland, OH).

Chemicals. Acetylcholine chloride was obtained from RBI. Anisomycin, Lavendustin A and B were obtained from Sigma.

Statistics.

The program GB-stats was used for statistical analysis. Student's t-Tests (multiple t-Test with Bonferroni adjustment) were performed to determine the significance of differences.

Results

Trophic factors regulate a wide range of cellular functions including outgrowth, synapse formation and plasticity (Davies, 1994; Markus et al., 2002; McAllister, 2002; Boyd and Gordon, 2003; Marzella and Gillespie, 2002; Huang and Reichardt, 2001; Yamamoto et al., 2002; Woodin et al., 1999, 2002). These effects are generally mediated through receptor tyrosine kinases and involve both gene transcription and *de novo* protein synthesis. However, the underlying mechanisms have yet to be elucidated. Here we investigate the effects of trophic factors on and the occurance of local protein synthesis in axonal sprouting as well as in AChR and ion channel expression, which constitute some of the axons intrinsic membrane properties.

Isolated LPeD1-axons contain components of the translation machinery.

To investigate whether isolated axons harbor components of the protein synthesis machinery, isolated LPeD1-axons were cultured (Figure 1A) and incubated in Syto14, which labels RNAs in addition to nuclear DNA. We observed that the dye indeed labeled the LPeD1-axon (Figure 1B,C), indicating that the soma-less axon contains RNA. Whether this is rRNA, tRNA or mRNA is not clear. Furthermore, EM investigation on

outgrowing cultured LPeD1 showed the presence of ribosomes in the neurites (Figure 1D).

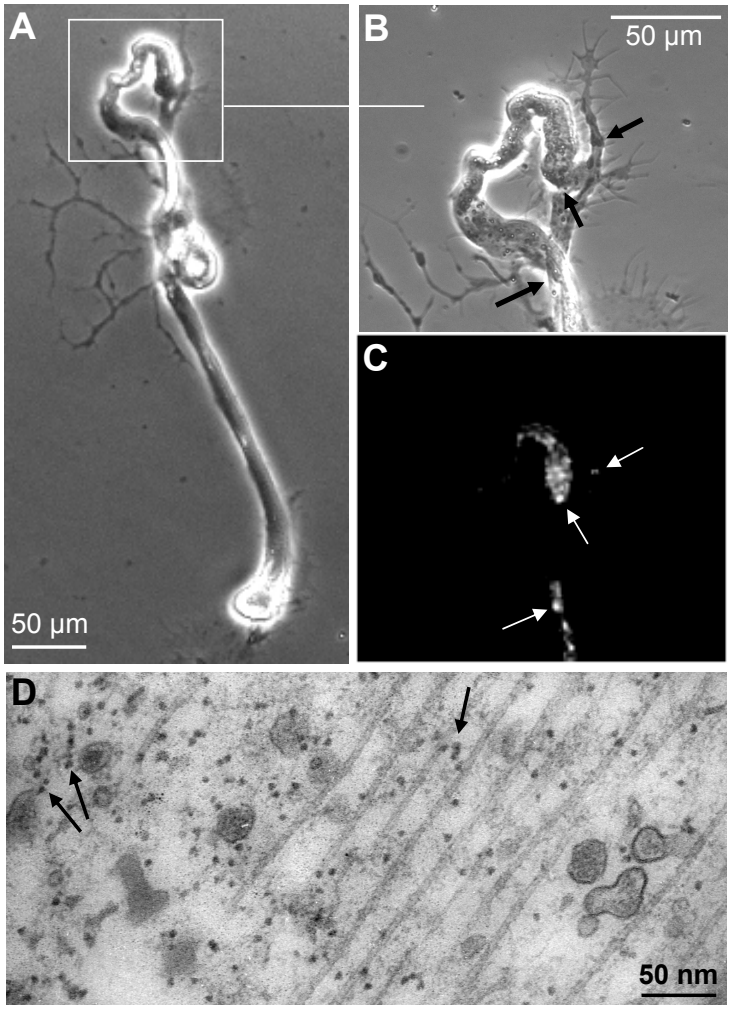

Figure 1: Isolated LPeD1-axons contain components of the translation machinery.
To determine whether isolated LPeD1-axons contain components of the protein synthesis machinery, axons were isolated in culture and incubated with Syto14, which stains all RNAs and DNA. (**A**) A single isolated LPeD1-axon, magnified in (**B**) shows punctate Syto14 staining (**C**), indicating the presence of RNA. The location of the white arrows in Figure C corresponds with the location of the black arrows in figure B, and indicates where the fluorescent spots are in the isolated axon. (**D**) Electron microscopy image of a LPeD1 neurite containing ribosomes. The arrows indicate clusters of ribosomes, suggesting the synthesis of proteins.

Considering these data, together with other studies on Lymnaea neurons showing local protein synthesis capabilities (Spencer et al., 2000; van Minnen and Syed, 2001), it can be suggested that LPeD1-axons contain components of the protein synthesis machinery and could therefore be capable of synthesizing proteins locally.

CM induced sprouting from isolated axons does not depend on protein synthesis.
To investigate whether trophic factors induce sprouting in isolated axons, isolated LPeD1 axons were maintained either in the presence or absence of CM. We observed that the isolated LPeD1-axons in CM exhibited characteristically different morphology than their DM counterparts. For instance, axons in CM extended elaborate neurites (Figure 2A), whereas, the growth in the DM group showed extensive veiling (Figure 2B). These results are comparable to other studies where neurite outgrowth from intact neurons was also shown to require trophic factors in CM (Rabacchi et al., 1999; Munno et al., 2000; Hermann et al., 2000).

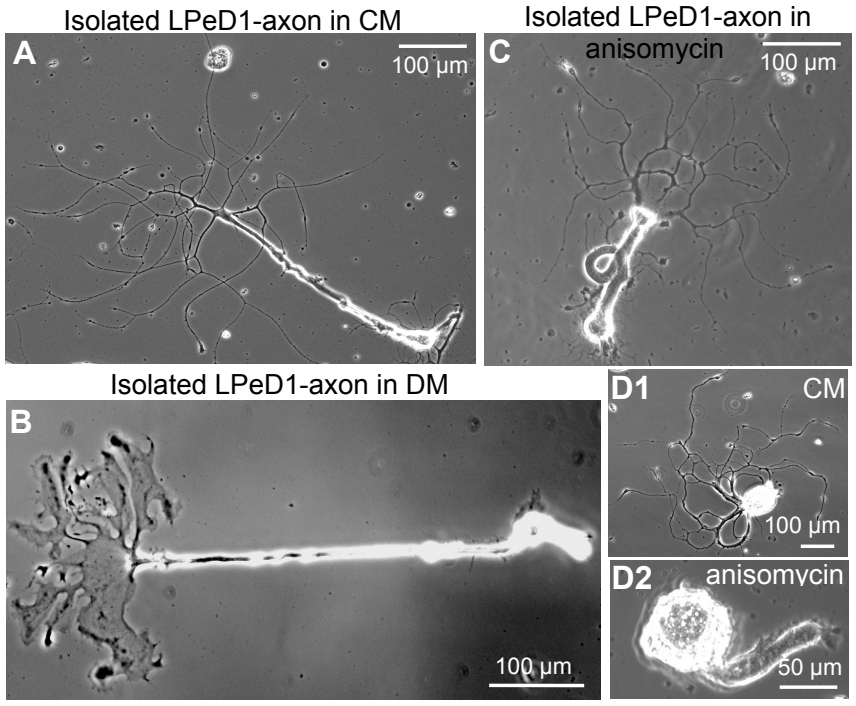

Figure 2: Isolated LPeD1-axons require trophic factors, but not protein synthesis, for sprouting.
(**A**) Single isolated axons cultured in CM exhibited extensive sprouting within 24 hrs. (**B**) To test whether this sprouting relied on trophic factors, single isolated LPeD1-axons were cultured in DM. Axons cultured under these circumstances did not exhibit sprouting, however, membranes still emerged from axons without forming distinct neurites. (**C**) Isolated axons cultured in the presence of anisomycin (12.5 µg/ml) were not affected by this protein synthesis inhibitor and exhibit normal sprouting. (**D1**) Contrastingly, LPeD1 soma cultured in CM exhibits sprouting, whereas (**D2**) LPeD1 soma cultured in CM+anisomycin (12.5 µg/ml) did not.

These data thus demonstrate that trophic factors can act locally to induce neurite outgrowth from the isolated axons. These data do not however reveal whether trophic factor-induced activation of receptor tyrosine kinases results in assembly of existing proteins or that the *de novo* synthesis underlies sprouting.

To investigate the involvement of *de novo* protein synthesis in trophic factor-induced sprouting, axons were cultured in CM either in the presence or absence of protein synthesis inhibitor anisomycin (12.5 μg/ml). After 24 hrs in culture, anisomycin did not significantly affect CM-induced neurite outgrowth from cultured LPeD1 axons (Figure 2C). In contrast, LPeD1 somata cultured in CM exhibited extensive sprouting (Figure 2-D1), whereas in CM + anisomycin (12.5 μg/ml) LPeD1 somata did not (Figure 2-D2). These results indicate that CM-induced sprouting in isolated axons is mediated by protein assembly and is independent of *de novo* protein synthesis.

Effect of trophic factors on intrinsic membrane properties of isolated axons.

We next determined whether trophic factors influenced intrinsic membrane properties of isolated axons. Axons were cultured overnight either in the presence or absence of CM and their intrinsic membrane properties (resting membrane potential, spiking threshold, action potential amplitude, and spontaneous activity) were analyzed. Even though the axons under both experimental conditions exhibited similar resting membrane potentials and spiking thresholds (Table 1), their ability to generate sustained spiking activity was severely compromised in DM. Specifically, current injections (0.2-0.5 nA) into axons maintained in CM resulted in sustained spiking, in a manner similar to that seen in the intact soma. However, axons maintained in DM often failed to sustain spiking in response to a constant depolarizing current pulse, which is also indicated by their reduced spontaneous activity compared to axons cultured in CM.

Next, the effects of local protein synthesis on the axons intrinsic membrane properties were investigated by inhibition of the synthesis of proteins. Isolated axons were cultured in the presence of anisomycin and tested for spontaneous activity, resting membrane potential, spiking threshold and action potential amplitude. Similar to the results obtained in DM and CM, anisomycin had no effect on spontaneous activity, RMP or spiking threshold. Interestingly, the action potential amplitudes of the axons cultured in anisomycin were significantly smaller than their counterparts in CM or DM without the inhibitor (Table 1).

Taken together, the above data demonstrate that trophic factors present in CM may regulate axonal sprouting, whereas they do not significantly regulate intrinsic membrane properties within the first 24 hrs of culture. Furthermore, the CM-induced sprouting does not require *de novo* protein synthesis for the initial 24 hrs, neither does the maintenance of resting membrane potentials and spiking thresholds. The action potential amplitudes of isolated LPeD1-axons, on the other hand, are affected by inhibition of local protein synthesis. Reason for this effect could be reduced ion channel expression.

Trophic factors alter the responsiveness of LPeD1-somata but not of isolated axons to exogenously applied ACh

To test for the involvement of trophic factors in synapse formation and synaptic plasticity, we next sought to define the mechanisms by which CM mediates excitatory, cholinergic response in LPeD1. Specifically, previous studies (Hamakawa et al., 1999;

TABLE 1A
INTRINSIC MEMBRANE PROPERTIES OF ISOLATED LPeD1-NEURONS

	Culture Condition	RMP (mV) Mean ± SEM	Spiking Threshold (mV) Mean ± SEM	Action Potential Amplitude (mV) Mean ± SEM	Spontaneous activity in % of axons	n
LPeD1-axon	CM	-60.90 ± 2.59	-54.54 ± 1.84	77.84 ± 0.78	73	11
LPeD1-axon	DM	-62.85 ± 5.32	-50.00 ± 1.54	78.12 ± 0.64	29	7
LPeD1-axon	CM + anisomycin	-55.00 ± 2.67	-51.78 ± 2.43	71.69 ± 0.59	64	14

TABLE 1B

p-values for RMP	Axon in CM	Axons in DM	Axons in CM + anisomycin
Axons in CM	----------	0.77	0.14
Axons in DM	0.77	----------	0.21
Axon in CM + anisomycin	0.14	0.21	----------

p-values for Spiking Threshold	Axon in CM	Axons in DM	Axons in CM + anisomycin
Axons in CM	----------	0.09	0.37
Axons in DM	0.09	----------	0.55
Axon in CM + anisomycin	0.37	0.55	----------

p-values for Action Potential Amplitudes	Axon in CM	Axons in DM	Axons in CM + anisomycin
Axons in CM	----------	0.78	<0.0001
Axons in DM	0.78	----------	<0.0001
Axon in CM + anisomycin	<0.0001	<0.0001	----------

Table 1: Intrinsic membrane properties of isolated LPeD1-axons under various culture conditions.
Table 1A: The intrinsic membrane properties resting membrane potential (RMP), spiking threshold, and action potential amplitude of isolated axons were evaluated under various culture conditions (CM, DM, and CM+anisomycin). To determine the mean amplitudes of action potential, multiple measurements were taken from each axon.
Table 1B: The p-values of resting membrane potentials (RMP), spiking thresholds, and action potential amplitudes show differences between these properties of LPeD1-axons cultured in the conditions displayed in table 1A. Multiple t-Tests with Bonferroni adjustment were performed to determine the level of significance under various conditions.

Woodin et al., 1999) have shown that the trophic factors are required for excitatory but not the inhibitory synapse formation between paired VD4 and LPeD1. Preliminary data from our lab also shows that the excitatory synapse formation may involve CM-induced insertion or maintenance of excitatory, cholinergic response elements in LPeD1 (Van et al., in preparation). Although LPeD1 and VD4 neurons paired in a soma-soma configuration developed either excitatory or inhibitory synapses in CM or DM, respectively, we found that VD4-soma and LPeD1-axon pairing in either CM or DM only resulted in excitatory synapse formation and that the inhibitory synapses were never observed. However, the incidence of excitatory synapse formation between soma-axon pairs was significantly reduced in DM (12%, n=25) as compared with its CM counterpart (100%, n=50) (see chapter 3). Thus, to define and compare the nature of their differential responsiveness to exogenously applied ACh, LPeD1-somata were cultured either in CM or DM alone. VD4's transmitter ACh was applied exogenously on the somata and the effects were tested via direct intracellular recordings. LPeD1-somata cultured in CM (Figure 3A), or DM (Figure 3B) exhibited excitatory and inhibitory responses, respectively, to exogenously applied ACh (1 μM). These results indicate that the trophic factors are required for the excitatory responsiveness of LPeD1-somata, and that in their absence the inhibitory response predominates.

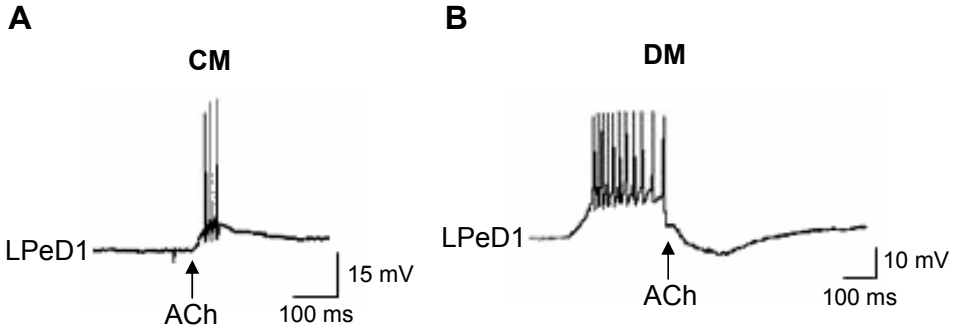

Figure 3: LPeD1-somata require trophic factors to alter responsiveness to exogenously applied ACh. In order to test the effects of trophic factors on LPeD1-somata and their responsiveness to ACh, somata of these specific neurons were cultured in the presence or absence of trophic factors. Subsequently, LPeD1-somata were exposed to local application of ACh (1 μM) and the effects were recorded electrophysiologically. (**A**) LPeD1-somata cultured in CM exhibited an excitatory response to locally applied ACh, which generated action potentials in these somata (n=5). (**B**) Somata cultured in DM exhibited an inhibitory response to local application of ACh, resulting in inhibited spiking and membrane hyperpolarization (n=5).

Next, the effect of trophic factors on the axon's responsiveness to exogenously applied ACh was tested. To test whether the reduced incidence of excitatory synapse formation in DM could also be attributed to a differential responsiveness of the isolated LPeD1-axons to ACh, this transmitter (1 μM) was pressure applied to isolated axons cultured either in CM or DM. Intracellular recordings revealed that axons cultured in either CM or DM responded in the same way to ACh. That is, under both circumstances

(n=9, Figure 4A for CM; n=8, Figure 4B for DM), ACh induced excitatory responses in these axons generating action potentials. Moreover, the involvement of local protein synthesis and receptor tyrosine kinase activation in excitatory AChR expression was tested as well. LPeD1-axons were cultured in the presence of anisomycin (12.5 µg/ml) to inhibit local protein synthesis, and in the presence of Lavendustin A (10 µM) to inhibit receptor tyrosine kinase activity, and its inactive analogue Lavendustin B (10 µM) was used as a control. The axonal responses to exogenously applied ACh were subsequently tested. As observed for axons cultured in CM and DM, axons cultured in anisomycin (n=6), Lav A (n=7) and Lav B (n=7) displayed normal excitatory responses to pressure applied ACh (Figure 4C), suggesting that, in contrast to their somata, the isolated axons of LPeD1 neurons do either not express inhibitory receptors, or trophic factors do not modulate the axons cholinergic responsiveness through either RTK activation or local protein synthesis.

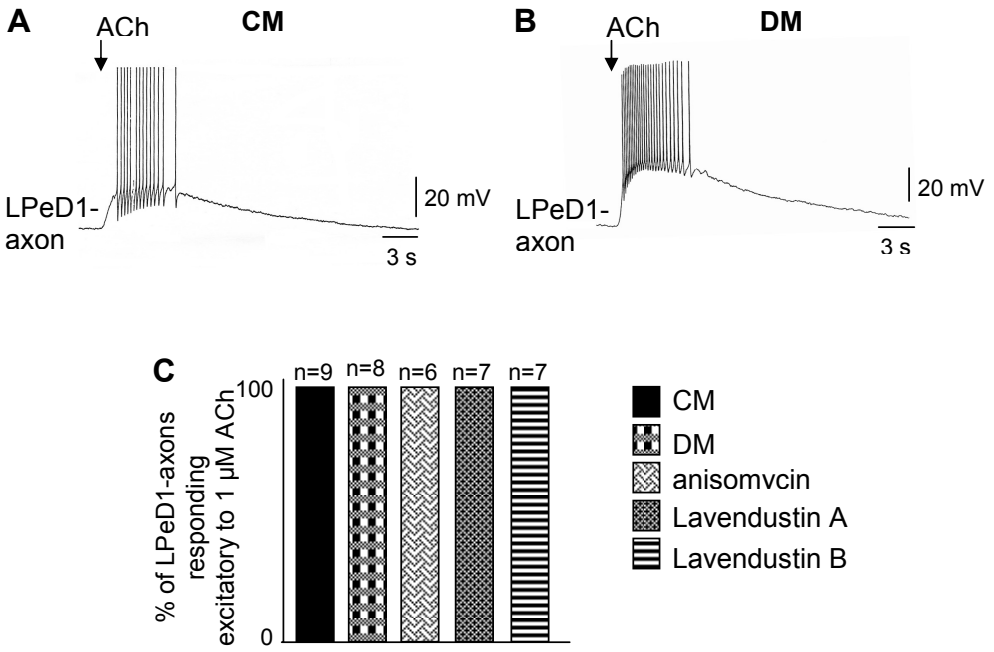

Figure 4: Trophic factors do not alter the responsiveness of isolated LPeD1-axons to exogenously applied ACh.
LPeD1-axons were cultured either in CM or DM and their responsiveness to exogenously applied ACh was determined. (**A**) Axons cultured in CM exhibited an excitatory response to locally applied ACh (1 µM) (n=9). (**B**) Similarly, axons cultured in DM exhibited excitatory responses to exogenously applied ACh (n=8). The involvement of local protein synthesis and receptor tyrosine kinase activation in the axons responsiveness was also tested. (**C**) Axons were cultured in the presence of either anisomycin (12.5 µg/ml) (n=6), Lavendustin A (10 µM) (n=7) or its inactive analogue Lavendustin B (10 µM) (n=7). Application of ACh evoked excitatory responses in all axons, regardless of the culture condition and the inhibitor present, suggesting that the excitatory responsiveness of LPeD1-axons to exogenously applied ACh is independent of trophic factors, protein synthesis and receptor tyrosine kinase activation.

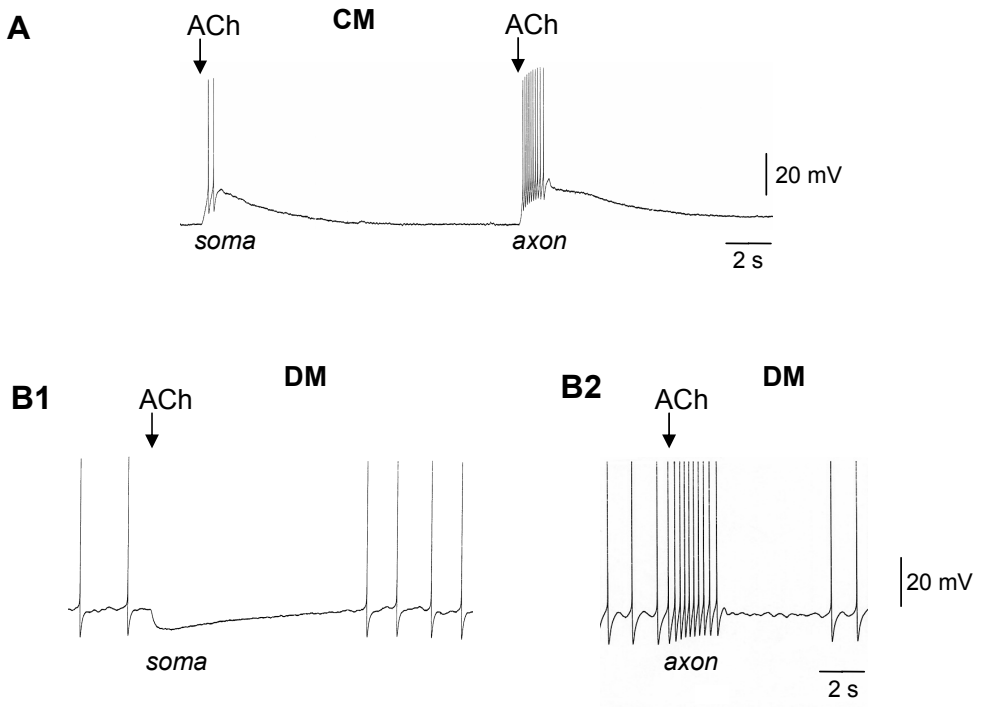

Figure 5: Differential receptor expression at the LPeD1-soma and the LPeD1-axon.
To test whether somata and axons of intact LPeD1 neurons display identical membrane properties upon ACh application, intact LPeD1 neurons were cultured either in CM or DM, and the effect of exogenously applied ACh was tested. Specifically, ACh was applied locally at the soma and at the axon. (**A**) In CM, 67% of the intact LPeD1 neurons exhibited excitatory responses to ACh, both at the soma and at the axon (n=14). (**B1**) However, in DM 100% of the intact LPeD1 neurons exhibited an inhibitory response to ACh applied at the soma (n=8), (**B2**) whereas ACh applied at the axon elicited and excitatory response (n=8). These data indicate that somatic and axonal regions of LPeD1 neurons express different sets of receptors in DM. In CM soma and axonal regions of LPeD1 neurons express similar or identical sets of ACh receptors.

Cholinergic receptors are differentially expressed on intact LPeD1-somata and their isolated axons.

The above results suggest that LPeD1-somata and axons may express different AChRs under identical culture circumstances. That is, in CM both soma and axon may exhibit an excitatory response to exogenously applied ACh, whereas in DM the somatic, but not the axonal excitatory response disappears. To rule out that this intra-cell difference in excitability is due to perturbed transport of somatic synthesized receptors that mediate inhibitory responses into the axonal region, we opted to test ACh on both the somata and axons of intact neurons. LPeD1 neurons were isolated with a long portion of their axon intact and cultured in either CM or DM. Under both culture conditions, ACh was applied exogenously on the soma and on the axon separately. In CM, 67% of the neurons exhibited an excitatory response to ACh both at the soma and the axon (n=14) (Figure 5A), 24% of the neurons exhibited an inhibitory response at the soma and an excitatory

response at the axon (n=5), whereas 9% of the neurons did not respond at all at the soma and responded excitatory at the axon (n=2). In other words, all the axonal compartments of the neurons exhibited an excitatory response to exogenously applied ACh (100%, n=21), whereas the responses of the somata of the same neurons displayed a variety of responses to ACh in that 67% responded excitatory (n=14), 24% inhibitory (n=5), and 9% did not respond at all (n=2). In DM, ACh induced inhibitory responses in the somata of all plated neurons (n=8, Figure 5-B1), whereas the axons of the same neurons exhibited only excitatory responses (n=8, Figure 5-B2). These data suggest that the cholinergic receptors are differentially expressed in LPeD1-somata versus LPeD1-axons, and that the inhibitory receptors are either not targeted to or located in the axons.

Discussion

In this chapter, we have demonstrated that for the first 24 hrs in culture, trophic factors play an important role in neurite outgrowth. The intrinsic membrane properties of isolated *Lymnaea* LPeD1 neurons were not affected by the presence or absence of trophic factors. Similarly, neurite outgrowth and intrinsic membrane properties, with the exception of action potential amplitudes, did not depend on local *de novo* protein synthesis.

The dependency of neurite outgrowth on trophic factors is consistent with earlier observations where BDNF was found to be important for locally regulating axonal and dendritic arborization (Lom and Cohen-Cory, 1999). Specifically, the dendritic arbor complexity of *Xenopus* retinal ganglion cells (RGC) is decreased by BDNF, whereas in the tectum BDNF increases RGC axonal arborization (Menna et al., 2003). Similarly, BDNF promotes regenerative sprouting in the serotonergic (5-HT) neurons in the adult rat brain (Mamounas et al., 2000). Other neurotrophic factors such as NT-3 and NT-4 also exert growth-promoting effects on developing neurons. Neural plate explants exhibit an increased number of neurites in the presence of NT-3 (Li and Bernd, 1999) and postnatal rat myenteric neurons increases the proportion of neurons as well as the number of cells extending processes (Saffrey et al., 2000). NT-4 has more variable effects on neuronal outgrowth. In cultured hippocampal neurons NT-4, in contrast to NT-3 and BDNF, did not promote extensive axonal outgrowth (Labelle and Leclere, 2000), whereas in retinal ganglion cells NT-4 induces extensive neurite outgrowth (Bosco and Linden, 1999). These studies, together with our results show that trophic factors can act locally and specifically and are capable of activating outgrowth in axons in the absence of soma based signaling. However, our results demonstrated that trophic factor-induced sprouting is not mediated by the activation of local protein synthesis. Axons cultured in CM+anisomycin exhibited a sprouting pattern that was not different from axons cultured in CM alone. Similarly, the maintenance of the axons intrinsic membrane properties for the first 24 hrs, except for action potential amplitudes, does not depend on the presence of trophic factors or on local protein synthesis. In contrast, other findings demonstrate local *de novo* protein synthesis in axons as well as dendrites regulating growth in the absence of the soma (Steward and Schuman, 2003). Reason for this discrepancy could be that the LPeD1-axons severed from their soma immediately after isolation from the brain where they already had established synaptic contacts, already contain a variety of proteins and

transcripts, which enables them to survive for a limited amount of time without the need of protein synthesis. In contrast, outgrowing neurons synthesize proteins while they extent axons and dendrites to find appropriate synaptic partners (Steward and Schuman, 2003). Since isolated LPeD1-axons are not in this developmental state, it renders them independent of local protein synthesis for the initial 24 hrs.

Although we have demonstrated that local protein synthesis is not necessary for axonal sprouting and intrinsic membrane properties, action potential amplitudes are affected by protein synthesis inhibition. Reason for this effect could be the increase, although not significant, of the RMP of axons in CM+anisomycin compared to normal CM. Specifically, in CM+anisomycin the RMP of axons is about 6 mV higher than that of axons in CM alone, and this increase reflects the decrease of action potential amplitudes in axons cultured in CM+anisomycin, which is about 6 mV as well. This increase in RMP can result in the shortening of the action potential amplitudes. Reason for the increase in the axons RMP could be loss of ion channels that are at the base of building up membrane potentials. Loss of ion exchangers (Na^+/K^+-exchangers) could have similar effects in that the re-establishment and maintenance of the RMP is slightly impaired. Since axons have been without local protein synthesis for only 24 hrs, the effects are not dramatic and have not yet impaired the axons integrity in a severe manner. Since we also demonstrated that components of the protein synthetic machinery reside in the axons, it is conceivable that this machinery can be used to synthesize household proteins in order to maintain the axons neuronal integrity. Considering that axons harbor already household proteins at the time of isolation, inhibition of local protein synthesis for 24 hrs does not have to result in dramatic impairments yet.

Trophic factors also played a role in the regulation of the axonal excitability. Besides the protein synthesis inhibition-induced decrease in action potential amplitude, isolated LPeD1-axons in DM show a decreased ability to continue spiking activity compared to axons in CM. It is conceivable that the presence of trophic factors influenced the axons excitability by either promoting the expression of axonal ion channels or modulation of existing channels. Examples of trophic factors exerting their effects on ion channel expression are provided by studies demonstrating that the functional expression of large-conductance Ca^{2+}-activated K^+ currents is regulated by neurotrophins (Martin-Caraballo and Dryer, 2002). Specifically, the expression of Ca^{2+}-activated K^+ currents ($IK_{(Ca)}$) in chicken CG neurons is dependent on EGF and beta1-neuregulin (Subramony and Dryer, 1997). Similarly, the development of Na^+ currents is dependent on NGF (Grumolato et al., 2003; Vidaltamayo et al., 2002) as well as the development of Ca^{2+} currents in PC12 cells (Grumolato et al., 2003) and the increase of existing HVA Ca^{2+} currents in *Lymnaea* motoneurons (Wildering et al., 1995). Since the RMPs and spiking thresholds of axons cultured in CM and DM are not significantly different from each other, it is not clear which and how ion channels are affected by trophic factors. Instead, ion channel modulation could alter the axons excitability without affecting or altering ion channel expression. Modulation of ion channels (Rosenbaum et al., 2002; Wang et al., 2003; Zhang et al., 2002) as well as changes in neuronal firing patterns (Rothe et al., 1999) have been demonstrated to be under the influence of trophic factors. Together, these studies demonstrate that trophic factors exert a pronounced effect on the modulation of ion channels, and may thus influence excitability. Considering the results presented in this chapter, it is not clear whether CM induces changes in ion

channel expression or modulates the existing ones. Further research will be required to determine this in more detail.

In this chapter we have also shown that the regulation of neurotransmitter receptor expression in neuronal somata is dependent on trophic factors. Specifically, in DM LPeD1 somata express receptors that respond in an inhibitory manner to exogenously applied ACh, whereas in CM LPeD1 somata express different receptors that are excited by this transmitter. Other studies consistent with this notion demonstrate that nerve growth factor (NGF) treatment of rat primary neurons induces a transient increase in muscarinic AChRs (Eva et al., 1992). Another trophic factor, BDNF, has been found to enhance specific GABA(A) receptor subunit mRNA expression in cultured mouse cerebellar granule neurons (Bulleit and Hsieh, 2000). In the isolated axons, however, the situation appears to be different. Either in CM or in DM, LPeD1-axons express receptors that respond excitatory to exogenously applied ACh. Moreover, protein synthesis inhibition or receptor tyrosine kinase inhibition do not affect this excitatory responsiveness of the LPeD1-axons. This suggests that by the time LPeD1-axons are isolated from the brain, they already harbor specific receptors. In culture, these axons assemble and express neurotransmitter receptors on the surface independent of trophic activity, protein synthesis or receptor tyrosine kinase activity. These data suggest that nuclear signaling might be pivotal in regulating the type of the AChRs expressed on the somata and axons. In addition, differential distribution of AChRs in intact neurons that are cultured in DM demonstrate that inhibitory receptors are expressed and kept on the soma, whereas excitatory receptors are synthesized and subsequently targeted and shipped towards the extrasomal compartments. However, it can also be argued that axonal AChRs are under the influence of neither the soma nor trophic factors, since we show in this chapter that axons express excitatory AChRs regardless culture conditions or the presence of the soma. This suggests that excitatory AChRs are located in the axonal compartments at the time of isolation and that only somatic AChR expression is regulated by trophic factors. In support of this is that in CM excitatory receptors are expressed both on axons and the majority of somata. Differential receptor expression has also been shown in the midbrain dopaminergic nuclei where diverse nicotinic AChRs are expressed (Klink et al., 2001). The functional significance of this differential distribution might be that the neuronal properties can be differentially modulated both locally at the synapses and globally at the soma. This will allow a variety of inputs to influence neuronal properties in a unique manner. In the case of LPeD1 neurons in culture, lack of trophic factors could provide a signal that results in the expression of inhibitory receptors on the soma surface or a change of receptor subunits resulting in a change of receptor ion permeability. That neurons are capable of receptor subunit switching has been recently demonstrated by Heinen et al. (2004). The axon however does not respond in this way to the lack of trophic factors, suggesting that receptor functions are more dynamically controlled at the somata whereas the synaptic receptors may not exhibit such flexibility.

What do our data elucidate on the role of the isolated LPeD1-axon in synapse formation? We know that the soma of the presynaptic VD4 is required for synapse formation, since this part of the neuron harbors gene transcription and translation, induced by trophic factors. The results presented in this chapter show that trophic factors are required for local protein synthesis-independent axonal sprouting, however, that is as far as that their role appears to go. For the expression of AChRs, ion channels, and the

maintenance of RMP and spiking threshold, isolated axons seem self sufficient for the initial 24 hrs in culture. The soma of LPeD1 neurons is the compartment that regulates specific AChR expression and the targeting to specific neuronal domains. Therefore, the role of postsynaptic axons in synapse formation appears to be very limited in that there needs to be a redistribution of the axonal AChRs to align with the presynaptic active zone. The next chapter therefore, will deal with the activity and cell-cell contact induced re-distribution of AChRs for synapse formation between VD4-somata and LPeD1-axons.

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chapter

5

Target Cell Contact And Extrinsic Trophic Factor-Mediated Signaling Is Required For Synapse Formation Between *Lymnaea* Neurons

with Naweed I Syed

Abstract

The interactions between neurotransmitters and their receptors are important for axonal path finding, growth cone behavior and synapse formation. However, their precise roles in synapse formation remain unclear. In this chapter, we provide evidence that neurotransmitter-receptor interactions are necessary for excitatory synapse formation between soma-axon paired cells. In addition, trophic factors and specific cell-cell signaling were found to act in concert to assemble acetylcholine receptors (AChRs) at the developing synaptic site. During synapse formation, the isolated axons execute an intrinsic program to target their AChRs specifically at the contact site with only one presynaptic neuron (VD4), even when paired with more (VD4s). Intact postsynaptic (LPeD1) neurons, on the other hand, develop synapses with multiple presynaptic partners, provided that both VD4s contact different regions of the same cell (soma and axon). In conclusion, it appears that somata and axons have each their own intrinsic selection mechanism for selecting synaptic partners, regardless whether somata and axons are severed from each other or are still parts of an intact neuron.

Introduction

During synapse formation both pre- and postsynaptic neurons undergo dramatic morphological and molecular changes to make their future synaptic needs compatible with functional demands. These changes include, among the aforementioned in previous chapters, clustering of neurotransmitter receptors at the synaptic site. At the NMJ, AChRs are expressed on the muscle surface prior to the arrival of the motoneuron (Lin et al., 2001). Upon contact with the motor nerve, these receptors cluster at the contact site with the neuron. Similarly, GABAergic innervation in vertebrates and invertebrates induces the clustering of GABA(A) receptors at synaptic sites (Christie et al., 2002; Christie and De Blas, 2003; Gally and Bessereau, 2003). Conversely, the formation of mature nicotinic receptor clusters in chick ciliary neurons is disrupted when innervation by the preganglionic neurons is prevented. The postsynaptic neurons express low levels of nicotinic receptors, which are organized in developmentally regulated early-stage clusters (Kaiser et al., 2002). These studies show the importance of presynaptic neurons in inducing morphological changes in the postsynaptic cell.

The clustering of neurotransmitter receptors on postsynaptic membranes is regulated by a variety of factors and perhaps the best described among them is agrin, which induces AChR clustering at the NMJ (Campanelli et al., 1991; Meier et al., 1997). The receptor for agrin on the postsynaptic muscle membrane is MuSK, a receptor tyrosine kinase (Glass et al., 1996a; Glass et al., 1996b; Hoch, 1999). Agrin induces tyrosine phosphorylation of MuSK (Hopf and Hoch, 1998), and dimerization of MuSK in turn induces tyrosine phosphorylation and clustering of AChRs (Hopf and Hoch, 1998). MuSK is required for synapse formation at the NMJ (DeChiara et al., 1996) and has been found to associate with AChRs (Fuhrer et al., 1997; Zhou et al., 1999). In CNS neurons, neuronal activity-regulated pentraxin (Narp) (Tsui et al., 1996), not agrin, is the protein that can cluster postsynaptic receptors. Narp, an immediate early gene (IEG) product whose expression is regulated by synaptic activity (O'Brien et al., 1999) and is secreted

by spinal and hippocampal axons (Mi et al., 2002), induces AMPA receptor clustering, and when overexpressed in cultured spinal neurons, it increases the number of excitatory synapses (O'Brien et al., 1999). In addition, dominant-negative Narp expressed in axons suppresses AMPA receptor clustering (O'Brien et al., 2002). These studies indicate that the gluatamate synapses of the CNS and the cholinergic synapses of the NMJ each employ their own specific neurotransmitter receptor aggregating factors to induce postsynaptic differentiation.

Neurotransmitter-receptor interactions have also been shown to be important for the aggregation of the postsynaptic apparatus. Although at the NMJ the AChRs are already expressed in pre-clusters before the arrival of the presynaptic neurons (Yang et al., 2001; Lin et al., 2001; Arber et al., 2002), neurotransmitter-receptor interactions are still crucial for the maturation (Wan and Poo, 1999) and maintenance of functional synaptic AChR clusters (Akaaboune et al., 1999). In the CNS, neurotransmitter-receptor interactions have been shown to play critical roles as well. For instance, growing axons are capable of neurotransmitter release prior to contact with postsynaptic targets (Sun and Poo, 1987; Kraszewski et al., 1995), suggesting a signaling function for neurotransmitters. Further, during synapse formation, presynaptic electrical activity induces release of glutamate, which in turn promotes dendritic filopodial motility (Dailey and Smith, 1996; Lendvai et al., 2000; Wong et al., 2000). In culture, *Lymnaea* neurons have been shown to secrete dopamine, and exogenous application of this neurotransmitter in turn induces growth cone attraction in target cells while growth cones of the non-target cells collapse (Spencer et al., 1996, 1998, 2000). Later, it was shown that the dopamine releasing neuron right pedal dorsal 1 (RPeD1), when co-cultured with target and non-target cells, promoted the advance of target cell growth cones while inhibiting the advance of non-target growth cones and induced growth cone collapse (Spencer et al., 2000). Perturbation of neurotransmitter-receptor interactions in the CNS of vertebrates and invertebrates result in drastic developmental abnormalities (Lauder, 1993; Shuey et al., 1992; Voronezhskaya, 1990; Voronezhskaya et al., 1992; Goldberg and Kater, 1989; Tennyson et al., 1983). However, the role of neurotransmitter-receptor interactions in synapse formation could be limited to specific types of synapses, such as cholinergic (Akaaboune et al., 1999; Misgeld et al., 2002) or dopaminergic (Spencer et al., 2000) synapses, whereas they will have no effect on the development of another type of synapse, such as glutamatergic synapses (Verhage et al., 2000). Specifically, normal axonal pathfinding and formation of glutamatergic synapses was demonstrated in knock-out mice with a genetically perturbed secretory machinery (Verhage et al., 2000). Lack of the expression of Munc 18-1, a protein necessary for neurotransmitter secretion, in mice did not prevent, among other things, normal development of synapses. However, neurons went into apoptosis after establishing synaptic connections, suggesting that transmitter release is involved, not in the development of synapses, but in the maintenance of established connections (Verhage et al., 2000).

In addition to the intrinsic interactions between neurons, extrinsic trophic factors can also induce a variety of pre-and postsynaptic changes. For instance, trophic factors can enhance transmitter release or aggregation of neurotransmitter receptors. For example, in the rat visual cortex BDNF and NGF were shown to enhance depolarization-evoked transmitter release (Sala et al., 1998). Moreover, in *Xenopus* nerve-muscle cultures transmitter release was potentiated by manipulating one single BDNF-coated

bead localized at the presynaptic axon (Zhang and Poo, 2002), suggesting that this trophic factor may also affect neurotransmitter release. Similarly, trophic factors have also been shown to modulate the structures and functions of the postsynaptic neurotransmitter receptors. The role of trophic factors in receptor clustering has been demonstrated in hippocampal neurons where tyrosine receptor kinase B (TrkB) mediates the clustering of postsynaptic neurotransmitter receptors. TrkB is diffusely distributed over dendrites and somata of neurons cultured *in vitro*. Treatment with BDNF, the TrkB agonist, increased the number of NMDA receptor and GABA(A) receptor clusters and their synaptic localization. Conversely, down-regulation of TrkB activity was followed by a decrease in NMDA receptor and GABA(A) receptor clustering and their synaptic localization (Elmariah et al., 2004), suggesting that neurotrophic factor receptor TrkB plays an important role in neurotransmitter receptor clustering at synaptic sites. Similarly, trophic factors could activate their cognate receptors in the soma-axon model, mediating the re-distribution of AChRs to the synaptic area between VD4 and LPeD1-axons (Meems et al., 2003).

Taken altogether, it appears that neurotransmitter-receptor interactions, neurotrophic factors, and neuronal innervation, play important roles in receptor clustering and synapse formation. Because trophic factors influence transmitter release and therefore may affect transmitter-receptor interactions between the neurons, we hypothesized that a trophic factor-induced release of ACh from VD4 and subsequent activation of AChRs on LPeD1 may be sufficient for excitatory synapse formation. From the previous chapter, it has become clear that the isolated axons express AChRs both in the presence or absence of trophic factors. The fact that trophic factors are still required for excitatory synapse formation might indicate their importance for neurotransmitter receptor clustering during synapse formation. In this chapter, we demonstrate that AChR activation in the soma-axon model is necessary but not sufficient for excitatory synapse formation. In addition, physical contact specifically with the VD4 acts in concert with trophic factors during synaptogenesis to induce re-distribution of axonal AChRs to the synaptic site. During synapse formation, the isolated axons execute an intrinsic program to target their AChRs specifically at the contact site with only one VD4. Intact LPeD1 neurons, on the other hand, develop synapses with multiple presynaptic partners, provided that both VD4s contact different regions of the same cell (soma and axon). These results indicate that within a single neuron trophic factors have different effects on AChR expression and re-distribution. At the soma trophic factors induce expression of excitatory AChRs for excitatory synapse formation, whereas at the axon of the same neuron trophic factors are not required for the expression of excitatory AChRs, however, they are critical for the re-distribution of existing receptors to synaptic sites for excitatory synapse formation.

Materials and Methods

Animals. Lymnaea stagnalis were maintained at room temperature in a well-aerated aquarium containing filtered pond water. For experiments involving cell isolation, snails approximately 1-2 months old (shell length 18-20 mm) were used, while conditioned medium (CM) was prepared from 2-3 month old animals (shell length 25-30 mm).

Cell Culture. Neurons were isolated from the central ring ganglia and maintained in cell culture as described previously (Syed et al., 1990; Ridgway et al., 1991; Syed et al., 1999). Briefly, snails were anesthetized with 10% Listerine solution (ethanol, 21.9%; methanol, 0.042%) in normal *Lymnaea* saline [(in mM): 51.3 NaCl, 1.7 KCl, 4.0 $CaCl_2$ and 1.5 $MgCl_2$] buffered to pH 7.9 with HEPES. The central ring ganglia were then washed several times (3 washes, 15 min each) with normal saline containing antibiotic (gentamycin, 50 μg/ml). The central ring ganglia were then treated with enzyme (trypsin) followed by enzyme inhibitor (trypsin inhibitor) and pinned down at the bottom of a dissection dish. All procedures were performed under sterile culture conditions.

Conditioned medium (CM) was prepared by incubating gentamycin (20 μg/ml)-treated ganglia in Sigmacote-treated glass petri dishes, containing defined medium (DM, L-15; Life Technologies, Gaithersburg, MD; Special Order). DM consisted of serum free, 50% L-15 medium with added inorganic salts (in mM: 40 NaCl, 1.7 KCl, 4.1 $CaCl_2$, 1.5 $MgCl_2$, and 10 HEPES, pH 7.9) and 20 μM gentamycin. The ganglia were incubated in a humidifier for 3 - 4 days (Syed et al., 1999; Wong et al., 1981) and the resulting CM was frozen (-20^o C) until used.

The identified neurons (somata and initial axon segment) were isolated by applying gentle suction through a fire-polished, Sigmacote (Sigma, St. Louis, Mo.)-treated pipette. The isolated neurons were then plated on poly-L-lysine-pretreated glass coverslips (Ridgway et al., 1991) in either DM or CM. Axons were isolated by first plating the cell body along with its intact axon segment in cell culture and allowing to adhere to the poly L-lysine coated dish. The axon was then immediately severed from the cell body by using a sharp glass pipette, and the severed cell body was subsequently removed from the culture dish. Soma-axon synapses were prepared juxtaposing the soma to the isolated axon. Triples comprised of one isolated axon and two VD4s were prepared in the same way. Triples comprised of one intact LPeD1 neurons and two VD4s were prepared by isolating the LPeD1 with its axonal segment first. Subsequently two VD4s were paired with the soma and the axon, respectively.

Electrophysiology. Neuronal activity was monitored using conventional intracellular recording techniques, as described previously (Syed and Winlow, 1991). Glass microelectrodes (1.5 μm internal diameter; World Precision Instruments, Sarasota, FL) were filled with a saturated solution of K_2SO_4 (resistance, 20-40 MΩ). An inverted microscope (Axiovert 135; Zeiss, Thornwood, NY) was used to view the neurons, which were impaled by Narashige (Tokyo, Japan) micromanipulators (MM202 and MM 204). Amplified electrical signals (Neuro Data Instrument Corp.) were
displayed on a digital storage oscilloscope (PM 3394; Philips, Eindhoven, The Netherlands) and recorded on a chart recorder (TA 240S; Gould, Cleveland, OH).

Chemicals. Acetylcholine chloride was obtained from RBI. Hexamethonium chloride was obtained from Sigma.

Results

In the previous chapters, we have demonstrated that although trophic factors are not required for the expression of excitatory AChRs on LPeD1-axons, they do nevertheless play an important role in the formation of excitatory synapses. It has been demonstrated in some neuronal systems that neuronal activity (Kwong and Gu, 2000) and transmitter-receptor interactions (Lauder, 1993; Spencer et al., 1998) are critical for axonal path finding, target cell selection, neurotransmitter receptor clustering and specific synapse formation, whereas in other model systems transmitter release is not important for the development of synapses (Verhage et al., 2000). We therefore sought to determine the involvement of trophic factors, neurotransmitter-receptor interactions, and cell-cell contact in the synaptogenic process between VD4-soma and LPeD1-axon pairs.

Synapse formation between VD4 soma and LPeD1 axon requires transmitter receptor interactions

Synaptic transmission between VD4 and LPeD1 is cholinergic and is blocked by appropriate antagonists (Woodin et al., 2002). To test the hypothesis that transmitter-receptor interactions between soma-axon pairs are critical for cholinergic synapse formation, the soma-axon pairs were cultured in CM either in the presence or absence of AChR antagonist hexamethonium (100 μM). After 18-24 hrs of culture, the medium containing the antagonist was replaced with fresh solution and synapses were tested. Pairs maintained in CM alone developed normal synapses as has been described previously (Meems et al., 2003), whereas 73% of the VD4 soma and LPeD1-axon pairs cultured in CM + hexamethonium failed to develop excitatory synapses (Figure 1-A1). To rule out the possibility that the absence of excitatory synaptic transmission may be due to the fact that the cholinergic receptors were either desensitized permanently by the drug or that its chronic treatment had affected postsynaptic responsiveness to ACh, their cholinergic responses were tested. The axons of soma-axon pairs cultured in the drug (both single and paired/ synapses and no synapses) were tested for their ability to exhibit an excitatory response to exogenously applied ACh. Pressure application of ACh (1 μM) directly to the axons induced an appropriate excitatory response in all axons tested (n=7) (Figure 1-A2). Overall, the incidence of excitatory synapse formation between soma-axon pairs cultured under control conditions was 100%, whereas in hexamethonium only 27% of the pairs developed excitatory synapses (Figure 1B). These data thus demonstrate that transmitter-receptor interactions between VD4 soma and LPeD1 axon are critical for excitatory synapse formation.

Both growth factors and VD4 contact are required to alter the responsiveness of LPeD1-axons to exogenously applied ACh.

To test whether VD4 alters the cholinergic responsiveness of LPeD1 axon at its contact site in CM, ACh was pressure applied. When applied to a single LPeD1 axon at its both proximal and distal ends, we observed almost identical excitatory responses in the isolated axon (n=13) (Figure 2A). We next sought to determine whether postsynaptic axons paired with the presynaptic soma displayed differential responses to ACh at the synaptic compared to extra-synaptic site. Simultaneous intracellular recordings were first made to demonstrate synapses between VD4 soma and the LPeD1 axon (n=7) (Figure 2B

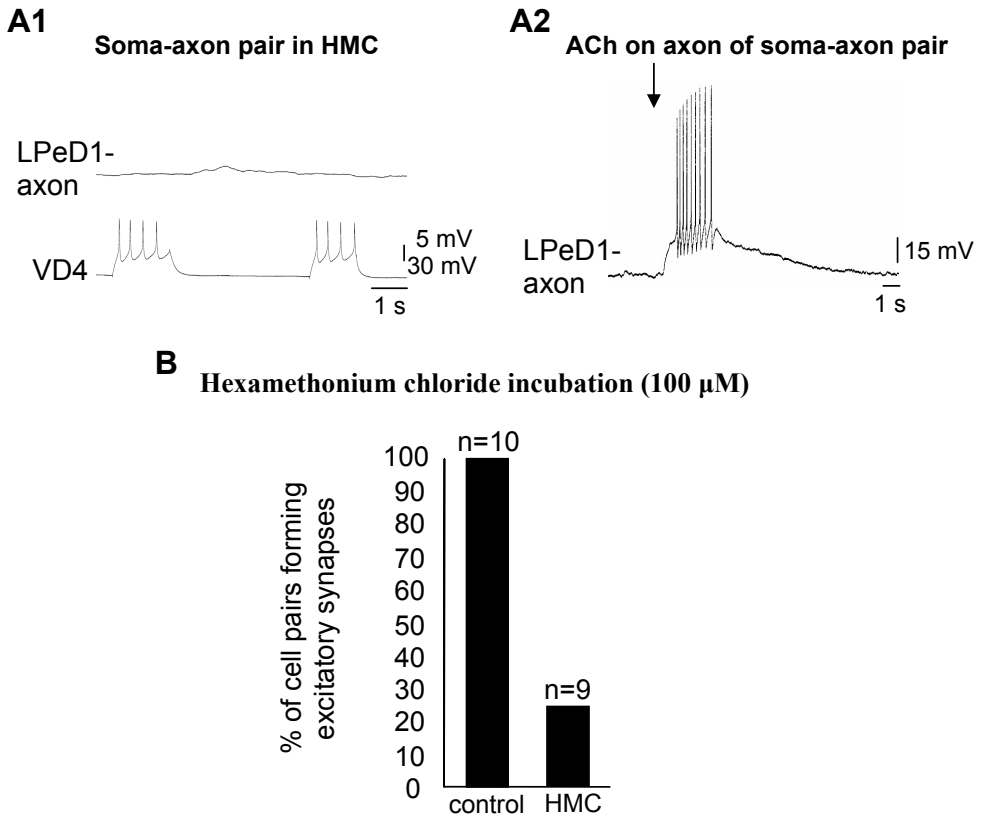

Figure 1: AChR antagonist inhibits soma-axon excitatory synapse formation.
To test the requirement of AChR activation in excitatory synapse formation, soma-axon pairs were cultured in the presence of the AChR antagonist hexamethonium chloride (HMC, 100 µM). (**A1**) Intracellular recordings show that excitatory synapses failed to develop in the presence of this antagonist, whereas (**A2**) the LPeD1-axon still responded in an excitatory manner to exogenously applied ACh (1 µM). Although synapse formation was not completely inhibited, 8 out of 11 of the soma-axon pairs failed to develop synapses, whereas the other 3 of the 11 pairs developed normal synaptic connections. These data are summarized in (**B**).

– *insert*). ACh (1 µM) was then tested (as above) for its effects at both the synaptic and extrasynaptic site (away from the contact). In all preparations (n=7) a single pulse of ACh applied directly at the contact site between VD4 and LPeD1 (axon) produced a strong excitatory response in the axon, which in most instances generated several action potentials (Figure 2B). Identical pressure application of ACh to the same axon, albeit at the extra-synaptic site, produced only small (~10 mV) sub-threshold, depolarizing responses in the paired axon (Figure 2C). These data demonstrate that, while both single and paired LPeD1 axons in CM respond to exogenously applied ACh, these responses however differ qualitatively in the paired axon at the synaptic versus extra-synaptic site. These results show that VD4 contact in CM induces ACh receptors to arrange such that

they selectively localize at the synaptic site. It is important to note that in all instances, axons were held at the same membrane potential (-58 mV).

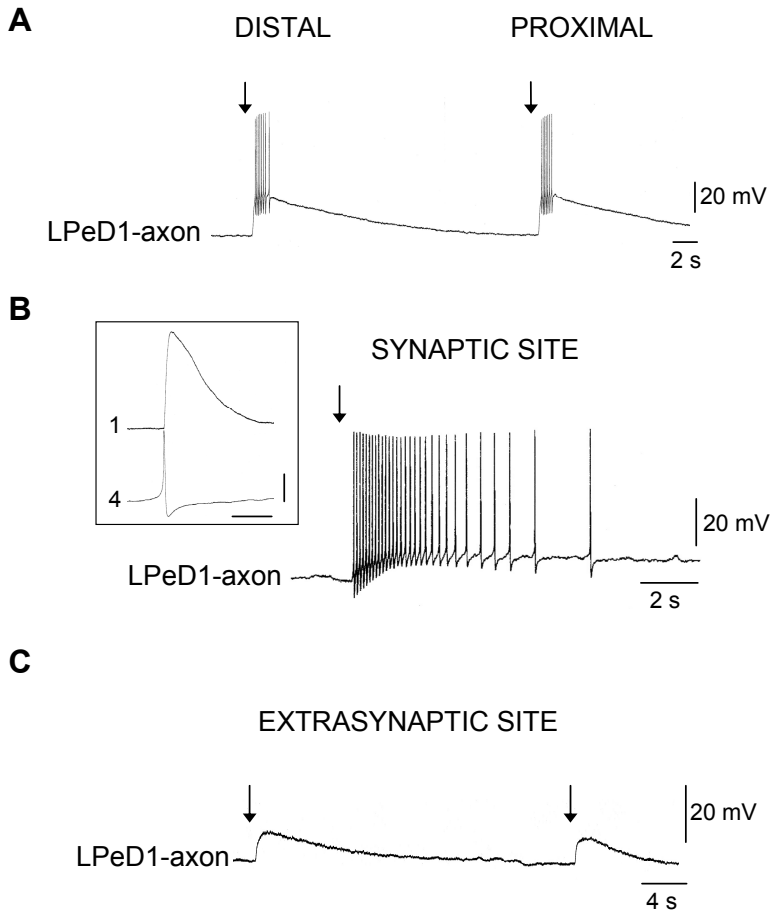

Figure 2: VD4 contact in CM changes the responsiveness of LPeD1-axons to exogenously applied ACh.
(**A**) A single LPeD1 axon maintained in CM exhibits excitatory responses to ACh. In all instances, ACh pulses applied at either distal or proximal site (see Figure A), generated action potentials in LPeD1 axon (n=13). A paired axon on the other hand, displayed differential responsiveness to exogenous ACh. Specifically, synapses were first demonstrated between the paired cells (**B-insert**; horizontal bar represents 1 second, vertical bar represents 5 mV for LPeD1 axon and 20 mV for VD4), where action potentials in VD4 (depicted in insert as 4) produced 1:1 EPSPs in LPeD1 axon (depicted in insert as 1). ACh was next tested at both synaptic (contacted with VD4) and extrasynaptic site (away from the contact point). (**B**) Whereas ACh pressure pulse at the synaptic site generated a strong burst of action potentials in LPeD1 axon , (**C**) only small depolarizing potentials were detected from the extrasynaptic site (n=7). All axons were held at a membrane potential of -58 mV.

VD4 contact in DM does not alter the responsiveness of LPeD1-axons to exogenously applied ACh.

To test whether VD4 contact with LPeD1 axon alone is sufficient to alter the responsiveness of LPeD1 axons to ACh, either single or paired axons were examined in DM. We found that both proximal and distal parts of a single isolated axon exhibited identical responses to exogenous ACh (1 μM) (n=7) (Figure 3A). Next, axons paired overnight with VD4 soma in DM were tested for their responsiveness to ACh. As shown

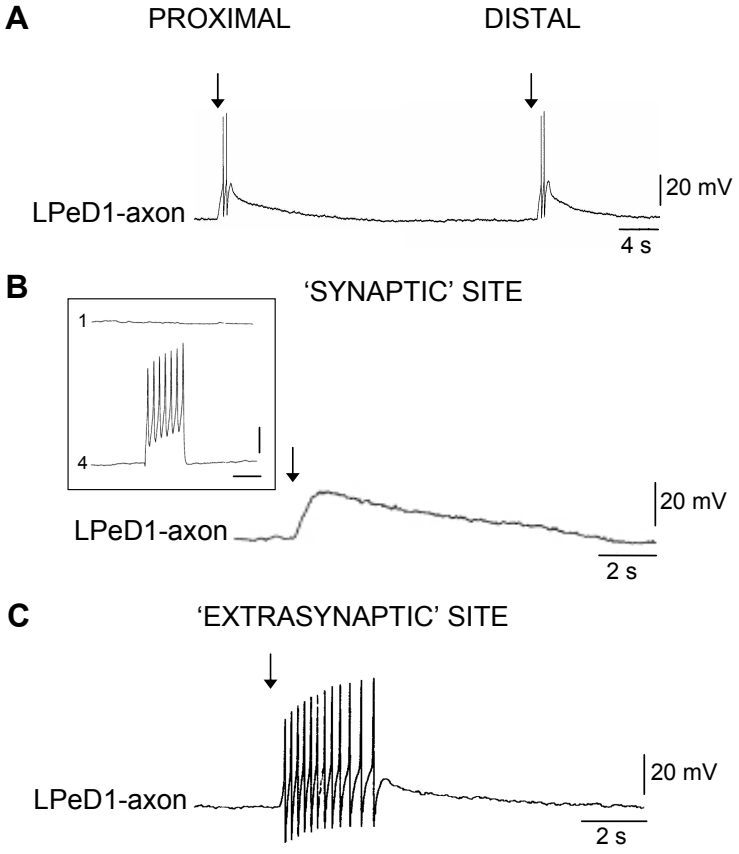

Figure 3: VD4 contact in DM does not change the responsiveness of LPeD1-axons to exogenously applied ACh.

(**A**) A single LPeD1 axon maintained in DM exhibits excitatory responses to ACh. In all instances, ACh pulses applied at either site (see figure A), generated action potentials in LPeD1 axon (n=8). VD4 soma/LPeD1 axon pairing in DM did not result in excitatory synapse formation (**B-insert**; horizontal bar represents 1 second, vertical bar represents 10 mV for LPeD1 axon and 20 mV for VD4) and a burst of action potentials in VD4 (depicted in insert as 4) failed to produce an excitatory response in LPeD1 axon (depicted in insert as 1). (**B**) Under these experimental conditions, ACh application at the contact site produced small depolarizing responses (which never generated spikes), (**C**) whereas ACh generated action potentials at the extrasynaptic site (n=8). All axons were held at a membrane potential of -58 mV.

earlier, no synapses were detected between the pairs in DM (n=25) (Figure 3B – *insert*). Eight out of 11 pairs tested under this experimental condition exhibited sub-threshold depolarizing responses to exogenously applied ACh at the contact site between VD4 and LPeD1 axon. However, the ACh-induced depolarizing responses rarely led to action potentials in the LPeD1-axon (Figure 3B). Identical pressure pulses of ACh at the non-contact site on the other hand, resulted in a burst of 10-12 action potentials in LPeD1 (Figure 3C). Taken together, these data demonstrate that VD4 contact in CM but not DM alters the responsiveness of LPeD1 axon to exogenously applied ACh. These results thus underscore the importance of trophic factors and transmitter-receptor interactions in mediating the cell-cell interactions that are essential for excitatory synapse formation between VD4 and LPeD1 axon.

A non-target cell contact does not alter the cholinergic responsiveness in LPeD1 axon

To test further the target cell specificity of cholinergic response in LPeD1 axon, we asked the question whether physical contact with a non-partner cell would be sufficient to alter its cholinergic responsiveness from contact versus non-contact site. To test this possibility, LPeD1-axon was paired with its non-synaptic partner visceral F cell, which contains the peptide FMRFamide (McKenney, 1992), (VF – *in vivo* located adjacent to VD4 but does not synapse with LPeD1) in CM and its cholinergic responsiveness was tested at both the contact versus non-contact sites. Intracellular recordings were made to demonstrate the specificity of synapse formation between VF and the LPeD1 axon. We found that VF cells did not form synapses with LPeD1 in all instances tested (n=6). Specifically, induced action potentials in VF cell did not affect the excitability of LPeD1 (either excitatory or inhibitory response) (Figure 4A-*insert*). Further, local application of ACh at the contact ('synaptic site') (Figure 4A) and the non-contact ('extrasynaptic site') (Figure 4B) revealed a stronger response at the non-contact (n=6) versus contact site (n=6) site. These results were in complete contrast with those observed in experiments where LPeD1 axon was paired with VD4 soma and thus indicate that a non-target cell contact does not alter the cholinergic responsiveness, which is both target cell and contact site specific.

Together, the data presented above demonstrate that VD4 contact with LPeD1 axon in CM induces receptor re-distribution such that they selectively localize to the contact site with the presynaptic cell. These effects require trophic factors and are target cell specific in that non-target cells do not induce the re-distribution of AChRs, possibly due to a lack of presynaptic acetylcholine release and the absence of specific membrane bound molecules that can function as specific recognition and re-distribution signals. If the receptors do indeed "cluster" at the contact site, then one would predict that a second VD4 paired with the LPeD1-axon would cause a double innervation of the same axon.

LPeD1-axons form only one synapse with any given VD4 neuron.

To determine LPeD1-axon's ability to form multiple synapses, it was simultaneously paired with two VD4s (Figure 5A). Intracellular recordings were made from both VD4 cells and the axon. We found that in all instances LPeD1-axons had permitted only one VD4 to establish synapses (Figure 5B+C), despite the physical contacts with both

neurons in CM. These data demonstrate that LPeD1 axon supports only a single VD4 neuron to establish synapses with it in CM.

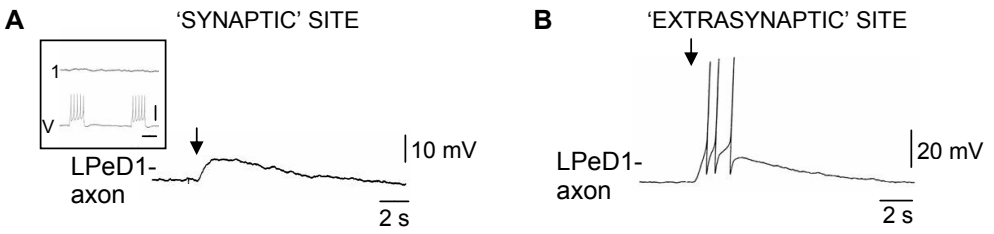

Figure 4: VF contact in CM does not alter the responsiveness of LPeD1-axons to exogenously applied ACh.
VF-soma/LPeD1-axon pairing in CM did not result in excitatory synapse formation (**A-insert**; horizontal bar represents 1 second, vertical bar represents 10 mV for LPeD1 axon and 20 mV for VF) and a burst of action potentials in VF failed to produce an excitatory response in LPeD1 axon (depicted in insert as 1). (**A**) Under these experimental conditions, ACh application at the contact site produced small depolarizing responses, (**B**) whereas ACh generated action potentials at the extrasynaptic site (n=6). All axons were held at a membrane potential of -58 mV.

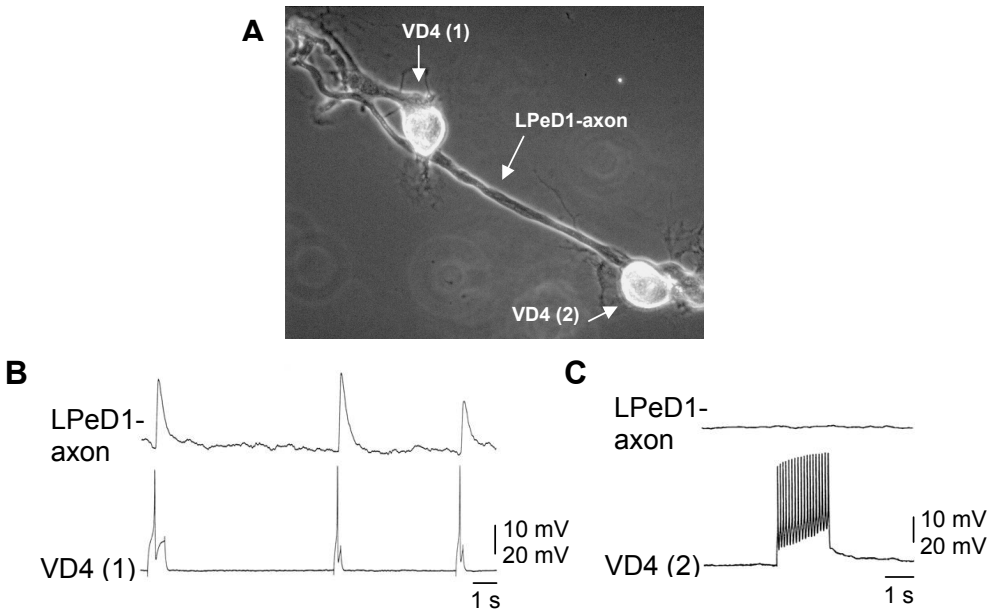

Figure 5: The LPeD1-axon forms a synapse with only one VD4.
To test whether LPeD1-axons would be able to form synapses with 2 VD4s, (**A**) single LPeD1-axons were cultured in CM and paired with a VD4 at either end of the axon. After 18-24 hrs intracellular recordings demonstrated that (**B**) if the axon had developed a synapse with one VD4, (**C**) synapses failed to develop between the axon and the other VD4.

From the above results, we postulated that LPeD1 axon permits synaptogenesis on a "first come first serve" basis and that the "preferred" cell attracts the majority of the receptors thus rendering the second VD4 devoid of sufficient receptor pool for an effective synaptic transmission.

Single LPeD1-axon, when severed in two halves, permits innervation by two VD4s.

To test whether a single LPeD1 axon severed in two halves would each exhibit similar cholinergic response allowing synaptic partnership to two different VD4 cells, LPeD1 was isolated along with its axon stump intact. After the soma removal, the axon was severed in two almost equal halves and maintained in CM overnight. On the second day, both halves were tested for their responsiveness to the exogenously applied ACh. Both halves of the LPeD1-axon exhibited identical excitatory responses to locally applied ACh (n=6) (Figure 6A+B), thus demonstrating an equal localization of ACh receptor pools. Based on these results, we thus postulated that each half of a severed LPeD1 axon would be able to synapse with two different VD4 cells.

To test this possibility, the LPeD1-axon was isolated and severed in two halves as described above. Each severed half was then paired with its corresponding VD4 neuron and the pairs were allowed to synapse overnight. Synapses were tested between VD4s and their corresponding axon segments. Interestingly, in most cases (n=8), synapses failed to develop in both 'axon-segment'/VD4 pairs. However, in a few cases where (n=5) synapses did develop in both pairs, the efficacy of synaptic responses was much weaker than that observed under normal conditions. In 2 cases one 'axon-segment'/VD4 pair developed an excitatory synapses whereas the other 'axon-segment'/VD4 pair did not exhibit any signs of synaptic transmission (n=2) (Figure 7). Taken together, these results suggest that AChRs are expressed in limited numbers on the axon surface and can thus afford innervation by only one VD4 for an effective transmission.

Because *in vivo* LPeD1 is innervated by the only VD4 cell that exists in the animal, we next asked whether an inability of the isolated axon to support two targets is a component of its intrinsic synaptic program or is caused by the inability to synthesize enough receptors to allow innervation by both cells.

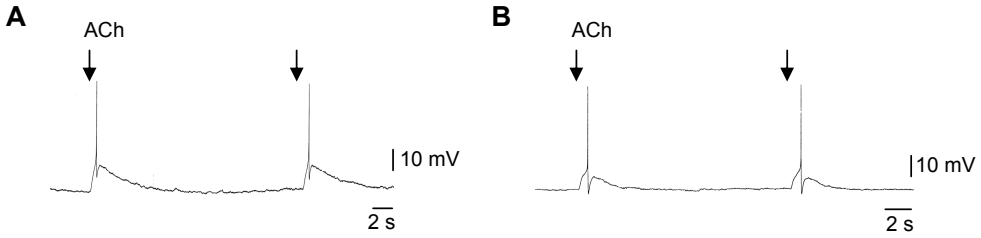

Figure 6: LPeD1-axons severed in two halves exhibited excitatory responses to exogenously applied ACh.
In order to determine whether LPeD1-axons severed in two halves still respond to ACh, these axons were cultured and parted in the middle. Local application of 1 μM ACh at each half evoked excitatory responses resulting in action potentials in both segments (**A**,**B**). Furthermore, the excitatory responses from both segments appeared similar, suggesting an even distribution of AChRs throughout the LPeD1-axon.

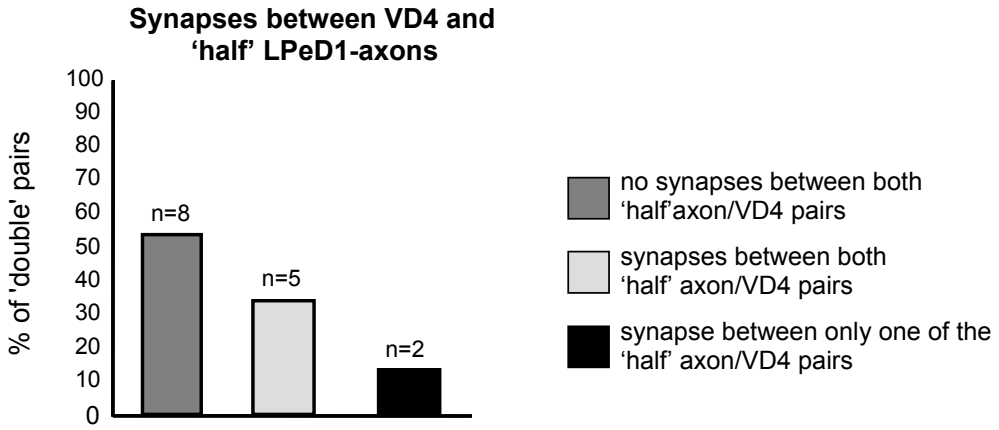

Figure 7: Synapse formation between VD4 and 'half' LPeD1-axons.
To test whether one LPeD1-axon parted in two segments would form synapses with VD4s paired with each segment, LPeD1-axons were cultured in CM and severed in two segments. Subsequently, VD4s were paired with each segment and synapses were tested. As shown in the diagram, in 8 of the 15 preparations both soma/axon-segment pairs did not develop synapses. In 5 of the preparations excitatory synapses were formed between both soma/axon-segment pairs, and in only 2 of the 15 preparations one soma/axon-segment developed a synapse whereas the other soma/axon-segment did not.

Intact LPeD1 in CM forms synapses with multiple VD4s.
To test the above two possibilities, a single intact LPeD1 was simultaneously paired with two VD4 neurons in CM overnight; one paired in a soma-soma and the other in a soma-axon configuration (Figure 8A) and synapses were tested electrophysiologically.

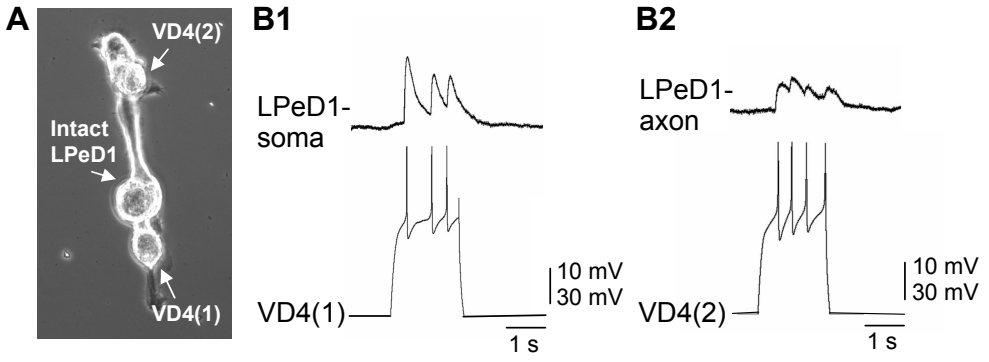

Figure 8: Intact LPeD1 forms excitatory synapses with two VD4s in CM.
To determine whether intact LPeD1 (soma and axon) neurons form synapses with multiple VD4s, LPeD1 neurons were isolated and plated in CM with the axonal segment. (**A**) After settling of the LPeD1, one VD4 was paired with the soma and the other with the axon. (**B1**) Intracellular recordings demonstrate mature excitatory synapses between the soma-soma as well as (**B2**) mature excitatory synapses between soma-axon (n=10).

Intracellular recordings revealed excitatory synapses between all of the soma-soma pairs (Figure 8-B1). Similarly, the second VD4 paired with the axon also developed excitatory synapses in all instances (Figure 8-B2) (n=6). These results indicate that with the soma intact, a LPeD1 neuron is able to synthesize enough receptors and can support innervation by the same target at multiple sites.

Discussion

In this chapter, we have demonstrated that excitatory synapse formation between VD4-soma and LPeD1-axon depends on AChR activation, possibly through the release of ACh from VD4. Blocking transmitter-receptor interactions in CM blocked synapse formation between the paired cells. We also showed that, as observed in the soma-soma model (Munno et al., 2003), isolated axons select only one specific presynaptic partner, which may be attributed to a limited number of receptors, thus limiting their ability to support multiple innervations. Moreover, the re-distribution of AChRs to synaptic sites requires the presence of trophic factors together with specific presynaptic target cell contact.

Using the soma-axon synapse, we have provided direct evidence that after blocking transmitter-receptor interactions between VD4 and LPeD1, the incidence of excitatory synapse formation is significantly reduced. It can be argued that a chronic treatment with AChR antagonist may have rendered the receptor incapable of function (desensitization, internalization, pore block etc), however, the axonal ability to respond to exogenously applied ACh speaks against this idea and strongly support our claim that blocking transmitter-receptor interactions did indeed block synapse formation between the cells. In support of the important role that neurotransmitters play, many studies in other animal models have shown that the synapse formation in the CNS was severely compromised when neurotransmitter-receptor interactions were perturbed during early development (Shuey et al., 1992; Goldberg and Kater, 1989). At a more cellular level it has been demonstrated that neurotransmitters are significant players in the assembly of the synaptic apparatus. For instance, in cultured rat spinal neurons glycine receptor (GlyR) activation is important for receptor clustering. Chronic treatment of these neurons with GlyR antagonist inhibited the accumulation of GlyRs at postsynaptic sites (Kirsch and Betz, 1998). In contrast, recent evidence demonstrating that neurotransmitter-receptor interactions are not critical for glutamatergic synapse formation was obtained from knock-out mice lacking munc 18-1. In these mice the regulated neurotransmitter secretion was completely abolished, whereas morphologically defined synapses developed normally (Verhage et al., 2000). Similar results were obtained at the NMJ, where normal acetylcholinesterase and AChR staining was observed in the endplate region of mutant muscle cells. Further, synaptic structures were also present at these mutant NMJs (Heeroma et al., 2003). In *C. elegans* GABA release is not essential for GABAR clustering at the NMJ. Specifically, GABAR clusters at GABAergic neuromuscular junctions developed normally in unc-25 mutants that do not synthesize GABA (Gally and Bessereau, 2003). However, the development of cholinergic NMJ synapses appears to require neurotransmitter-receptor interactions, since blockage of neurotransmission in living adult mice decreased ACh receptor number and density (Akaaboune et al., 1999). In addition, ChAT knock-out mice, in which neurotransmission

is blocked due to that lack choline acetyltransferase, demonstrated that neurotransmission affects, among other things, the number and distribution of synaptic sites, as well as the formation and stabilization of nerve-muscle contacts (Misgeld et al., 2002). Our data exhibits a striking resemblance with studies on cholinergic NMJ synapse formation and underscore the importance of transmitter-receptor interactions in the development of cholinergic synapse and demonstrate the importance of activity dependent mechanisms in directing AChRs to synaptic sites.

Mechanisms by which activated neurotransmitter receptor may be aggregated at synaptic sites are not well understood. Neurotransmitter receptor activation seems to be important for receptor clustering. For instance, in rat spinal neurons the accumulation of GlyR on the postsynaptic membrane is inhibited by a GlyR antagonist (Kirsch and Betz, 1998). It has also been demonstrated that neurotransmitter-receptor interactions lead to second messenger system activation such as Ca^{2+} and cAMP. In hippocampal interneurons, Ca^{2+} influx as well as Ca^{2+} release from intracellular stores is mediated by mGluRs (Woodall et al., 1999). The serotonin receptor 5-HT5A was found to mediate the transient opening K^{+} channels, possibly due to elevated intracellular Ca^{2+} levels, and the reduction of cAMP levels (Noda et al., 2004). Dopamine induces retraction of cultured retinal growth cones, which is accompanied by increased cAMP (Lankford et al., 1987; Lankford et al., 1988). Considering these effects of neurotransmitter receptor activation, it is conceivable to reason that neurotransmitter receptor-mediated activation of second messengers such as Ca^{2+} and cAMP could phosphorylate the activated neurotransmitter receptors or scaffolding proteins, such as PSD-95, rapsyn, and utrophin, which in turn could anchor neurotransmitter receptors to the cytoskeleton at synaptic sites.

Local neurotransmitter receptor stimulation is obviously achieved through neurotransmitter release by contacting presynaptic neurons. Trophic factors have been demonstrated to modulate neurotransmitter release in a variety of preparations. As shown in our soma-axon model in chapter 3, contact between pre-and postsynaptic neurons does not suffice to regulate synaptic development, suggesting the possibility that VD4 either does not secrete ACh in the absence of trophic factors or that its levels are significantly reduced. Consistent with this notion, BDNF has been shown to increase quantal neurotransmitter release at synaptic sites mediated by presynaptic mechanisms (Zhang and Poo, 2002). It has been proposed that neurotrophins target the vesicle retrieve mechanism at presynaptic sites. The likelihood that ACh release may not have occurred in DM is ruled out by the fact VD4 continues to make inhibitory synapses with LPeD1 which are, although inappropriate, nevertheless cholinergic in nature. In addition, 12% of LPeD1-axon and VD4-soma pairs in DM did form excitatory synapses. These data thus demonstrate that VD4's secretory machinery may not be affected by CM. So what would be the site where the trophic factor acts?

Trophic factor effects have been reported on the postsynaptic site. Trophic factors could exert their effects on axons through direct or indirect interactions with the existing AChRs. For instance, trophic factors could activate Trk receptors, which in turn recruit or activate scaffolding and anchoring proteins to the synaptic site. This could result in recruitment of otherwise dispersed AChRs to the contact site with the VD4. More directly, activated Trk receptors could interact with AChRs and anchor them at the postsynaptic zone. Although there is no evidence to show that direct interactions between Trk receptor and neurotransmitter receptors do indeed occur, neurotrophins have been

demonstrated to influence neurotransmitter receptor clustering by some unknown mechanisms (Elmariah et al., 2004). Specifically, activation of the trophic factor receptor TrkB mediates the clustering of neurotransmitter receptors at synaptic localizations. Treatment of these neurons with BDNF promoted an increase in NMDAR and GABA(A)R clusters on the postsynaptic terminals. Conversely, receptor cluster numbers and their synaptic localization were decreased through down-regulation of TrkB-mediated signaling (Elmariah et al., 2004). Similarly, in this chapter we have demonstrated that CM is required for synapse formation and that these trophic factors act in concert with presynaptic contact to induce receptor localization at the synaptic site. Besides the suggested direct effects, possible indirect trophic effects on isolated axons may be mediated by trophic factor-induced changes in the presynaptic VD4, which in turn promote the aggregation of the postsynaptic machinery. For instance, as mentioned in the introduction, Narp has been demonstrated to induce aggregation of neurotransmitter receptors in central neurons (O'Brien et al., 1999). In the soma-axon model, trophic factors could induce secretion of Narp homologues by the presynaptic VD4, providing a localizing signal for the AChRs on the LPeD1-axon resulting in the aggregation of these receptors at the contact site with the VD4. In addition, as mentioned in the previous paragraphs, transmitter-receptor interactions may play an important role in the aggregation of postsynaptic neurotransmitter receptors. Transmitter release or increase in transmitter secretion induced by trophic factors (Sala et al., 1998) could provide signals for the re-distribution of AChRs. Finally, trophic factors may also influence the expression of membrane bound proteins on the VD4 surface, which in turn could, for example, phosphorylate key proteins or induce local intracellular Ca^{2+} elevations in the axon, localizing the site of presynaptic contact.

In the intact brain only one VD4 is available to the LPeD1 neuron. In culture, however, it is possible to present neurons with multiple presynaptic partners. Isolated LPeD1-axons paired with two presynaptic VD4s demonstrated that the *in vivo* specificity of synaptic connections was maintained in cell culture. Neuronal ability to regulate the number and the efficacy of its synapses between soma-soma pairs has previously been demonstrated. Specifically, Munno et al. have demonstrated that neuronal cultures comprised of three somata, two LPeD1 somata paired with one VD4 soma, developed synapses between only one of the LPeD1s and the VD4 (Munno et al., 2003). However, in these triples it was shown that the number of synapses that developed was regulated by the presynaptic VD4. In our model, with one LPeD1-axon and two VD4 somata, it appears to be the postsynaptic LPeD1-axon that does the selection of its synaptic partner. Therefore, LPeD1, like VD4 neurons, may have an intrinsic selection mechanism that recruits the expressed AChRs to only one synaptic site. Another possibility is that LPeD1-axons may just have a limited number of receptors available that is just sufficient to effectively support innervation from only one VD4. Our data furthermore show that parted LPeD1-axons (although the incidence of synapse formation was only 33.3%) were capable of forming excitatory synapses with both parts of the same axon. Severing LPeD1-axon in half yielded two independent axons, and both formed excitatory synapses with their own paired VD4, since there is not competition between the VD4s. Interestingly though, the synapses that did form between parted axon segments and their paired VD4s did not exhibit the properties of a fully developed and mature synapse. This could be due to a limited number of receptors available. All together, these results

indicate that LPeD1 neurons are programmed to receive innervation from single VD4 – as would be the case in the intact brain, and that the LPeD1-axon has its own mechanism to ensure that no faulty or redundant synaptic connections are established.

Neuronal cultures composed of two VD4s and one intact LPeD1 (soma and axon) add an extra facet to the story. An intact LPeD1 neuron does form multiple synapses when presented with more than one presynaptic VD4, namely at the cell body as well as at the axonal domain. These data demonstrate that each neuronal compartment, the axon and soma of the LPeD1, is capable of forming one synaptic connection. Considering that *in vivo* the somata and axons of the same neuron can be located at some distance from each other, it seems likely that different compartments of the LPeD1 neuron are functioning more or less independently from each other.

In overview, the presynaptic VD4 transcribes and translates all the proteins required for the synaptic machinery. The LPeD1-axon, on the other hand, contains all the required components for synapse formation. However, these components are assembled at the synaptic site only after contact with the VD4 is established in the presence of trophic factors. Furthermore, neurotransmitter-receptor interactions are necessary but not sufficient for the development of excitatory synapses. In light of the previous chapter, where it was demonstrated that single, intact LPeD1 neurons display differential regulation of AChR expression at the soma versus the axon, the results from this chapter suggest that synapse formation could also be differentially regulated at the soma versus the axon. In addition, the fact that an intact LPeD1 neuron can form synapses at each different compartment indicates that different signals from different areas of the brain can be integrated in this single neuron. This demands a hierarchic order of synaptic organization between soma and axon within the neuron. Since the soma contains the nucleus for gene transcription and the machinery for protein synthesis, it has access to all required proteins. Alteration in neurotransmitter receptor expression resulting from environmental changes and subsequent alterations in synaptic properties, may therefore be executed at the soma only. The axonal compartments, on the other hand, are dependent on the various proteins or mRNAs shipped from the soma and are therefore restricted in their ability to modulate synaptic changes. Signals coming in at both the soma and the axon will nevertheless need to be integrated and the overall input will determine the neurons' responses and excitability. For instance, inhibitory synapses at the soma might override excitatory synapses at the axon, keeping the neuron hyperpolarized and thus blocking the local signals that may come from the axonal compartments, unless the excitatory signals coming in from the axon are strong enough to override the soma's inhibitory input. In this way it is not necessary for axons to change the nature of their synapse, since the overall response remains mainly determined by the soma in the context of synaptic integration at the level of functional neuronal circuits.

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General Conclusion

To study synapse formation in detail, at the level of a single pre-and postsynaptic neurons, the model system approach remains highly valuable and desirable. The pond snail *Lymnaea stagnalis* has proven to be a useful model for such studies because functionally well-defined neurons can be identified *in vivo* and their synapses reconstructed in cell culture. The ability of adult *Lymnaea* neurons to regenerate their axonal and synaptic connections is an added bonus, as one can avoid anatomical challenges of working with embryonic tissues. Notwithstanding the fact that *Lymnaea* model is not as suitable for genetic manipulations as its invertebrate counterparts such as the worm and the fly, it does nevertheless offer the advantage of being able to make direct intracellular measurements of synaptic physiology at a resolution not approachable in *C. elegans* and *drosophila*.

Another characteristic of *Lymnaea* neurons is their axons can function both structurally and functionally for some time in the absence of their cell body, thus allowing one to explore the role of various extrasomal compartments in synapse formation and synaptic plasticity. It appears that both pre- and postsynaptic somata are not required for the maintenance of already formed excitatory synapses. However, for new synapses to form the presence of the presynaptic but not the postsynaptic soma were found to be required. Further, presynaptic gene transcription and protein synthesis underlie the development of synapses, whereas postsynaptic protein synthesis did not play a role in this process, even though the components of the protein synthetic machinery are present in isolated axons.

Besides the role of intrinsic cellular processes in synapse formation, extracellular factors such as trophic factors have turned out to be indispensable for synaptic plasticity and synaptogenesis and these effects involve RTK activation in the pre- but not the postsynaptic cell. These data thus demonstrate the role that a presynaptic cell will play in its partnership with the postsynaptic cell during synapse formation. How trophic factors affect postsynaptic receptor re-location is an important issue that should be the focus of future work.

Cell-cell interactions are also crucial for the re-distribution of AChRs and synapse formation. Contact with specific presynaptic neurons induces the aggregation of AChRs at the contact site, whereas other (non target) neurons have no effect on the re-distribution of AChRs. Importantly, trophic factors will have to be present during contact, which indicates that cell-cell interaction and the trophic factors act in concert to aggregate AChRs at the synapse. Another critical step for synapse formation involves the interactions between neurotransmitter ACh and its receptors, since inhibition of neurotransmitter-receptor interactions perturbed synapse formation.

Postsynaptic neurons were found to regulate the number of innervation sites. Each neuronal compartment (somatic and axonal) forms only one synapse, even when presented with multiple presynaptic neurons. This could be due to a limited number of receptors. Since different parts of the postsynaptic neuron are able to form synapses simultaneously, a hierarchic order may be required when the types of synapses are different from each other (one inhibitory and the other excitatory). Since the soma contains the nucleus for gene transcription and the machinery for protein synthesis, this compartment may thus be the most adaptable to changes. Instead of altering the nature of synaptic connections at both the axon and the soma, only the latter would suffice to fine tune the synaptic output of a given cell. Synaptic signals received at the soma can

override the synaptic signals coming in at the axonal synapse. In this manner, the soma being at the top of the hierarchic order, determines the overall response and excitability of the whole neuron.

Nederlandse Samenvatting

Het centrale zenuwstelsel voert tal van fysiologische en cognitieve taken uit. Om deze op een efficiënte manier te kunnen laten verlopen, is het van belang dat de juiste synaptische connecties gevormd worden. De groei en ontwikkeling van het centrale zenuwstelsel bestaat uit migratie, cellulaire differentiatie, neuronale uitgroei, en het vinden van de juiste doelwitcel en de vorming van synapsen. Omdat de ontwikkeling van het zenuwstelsel uit zoveel fases en stappen bestaat, is deze kwetsbaar voor fouten voordat de neuronale netwerken compleet gevormd zijn. Het is daarom van belang dat alle ontwikkelingsstappen nauwkeurig geregeld zijn. Stoornissen in de normale functie van het centrale zenuwstelsel, zoals schizofrenie en de ziekte van Parkinson, zijn het resultaat van storingen in de ontwikkeling van zenuwcellen en synaptische connecties en van de daaruit voortkomende ontregelde vorming van neuronale netwerken. In dit proefschrift wordt één van de laatste stappen in de vorming van neuronale netwerken, de vorming van synapsen, nader bestudeerd.

In het centrale zenuwstelsel volgt de vorming van synapsen na de uitgroei van neuronen die hun weg vinden naar hun juiste synaptische partners. Neuronen ontwikkelen tijdens de uitgroei twee soorten neurieten, namelijk dendrieten en axonen. Axonen zenden signalen uit die worden ontvangen door dendrieten van naburige neuronen. De contactplaats tussen het axon van het ene neuron en de dendriet van het naburige neuron wordt de synaps genoemd. De eiwitten en andere componenten die nodig zijn om signalen over te dragen van axonen op dendrieten worden in het cellichaam gemaakt en naar de synaps getransporteerd. Hierdoor zijn synapsen, ook die ver verwijderd liggen van het cellichaam, afhankelijk van dit langeafstandstransport. De laatste jaren echter werd meer en meer de vraag gesteld hoe synapsen snelle plasticiteit vertonen terwijl ze afhankelijk zijn van eiwitten die soms over lange afstanden vanuit het cellichaam getransporteerd moeten worden. Aan de directe behoefte aan nieuwe eiwitten kan op deze manier niet worden voldaan. De locale synthese van eiwitten is dé oplossing voor dit dilemma. Het is thans aangetoond dat axonen in staat zijn om vele verschillende soorten messenger RNA (mRNA), sjablonen voor de vorming van eiwitten, te vertalen in eiwitten. Bovendien zijn ook de componenten van de eiwitsynthese machinerie gevonden in axonen, alsmede ook in synapsen. Synaptische plasticiteit is aangetoond afhankelijk te zijn van locale synthese en ook de vorming van synapsen lijkt deels afhankelijk te zijn van synaptische eiwitsynthese.

Naast interne factoren die invloed uitoefenen op de morfologie en fysiologie van neuronen zijn er ook externe factoren die een rol spelen. Neuronen staan bloot aan trophe factoren die invloed uitoefenen op diverse processen zoals neuronale uitgroei, differentiatie en ook de vorming van synapsen. Deze factoren kunnen een stimulerende, maar ook een remmende invloed hebben op de uitgroei van neuronen, afhankelijk van het type neuron en de trophe factor. Op dezelfde manier kunnen trophe factoren ook bepalen of een ontwikkelende synaps van excitatoire of inhibitoire aard is. Het mechanisme waardoor trophe factoren hun invloed uitoefenen wordt gemedieerd door receptoren in het cel membraan van neuronen. Deze receptoren worden tyrosine kinase (Trk) receptoren genoemd en bevatten een kinase enzym. Binding van trophe factoren aan hun Trk receptor activeert het kinase aan de intracellulaire kant van het celmembraan. De kinase activeert vervolgens andere processen in de neuron die onder andere van belang zijn voor de overleving, groei en de vorming van synapsen.

De zoetwaterslak *Lymnaea stagnalis* heeft jarenlang bewezen een goed model te zijn voor het onderzoeken van neuronale uitgroei, locale eiwitsynthese en de vorming van synapsen. Het voordeel van *Lymnaea* ligt in het feit dat het centrale zenuwstelsel eenvoudig is van opbouw vergeleken met dat van zoogdieren, de neuronen groter zijn en het makkelijk is om geïdentificeerde neuronen in kweek te brengen. Het is n.l. mogelijk om enkele neuronen uit de slak te isoleren en ze te laten uitgroeien en synapsen te vormen. Synapsen die aanwezig zijn in het intacte brein van de slak worden weer opnieuw gevormd in de kweek. Dit schept mogelijkheden om specifieke synapsen te onderzoeken en te manipuleren. Neuronen die vaak gebruikt zijn om de vorming van synapsen te bestuderen zijn de Visceral Dorsal 4 (VD4) en de Left Pedal Dorsal 1 (LPeD1). De synaps tussen deze twee neuronen in het intacte brein is een excitatoire synaps. In kweek vormen deze twee neuronen dezelfde excitatoire synaps. Om te voorkomen dat de uitgroei van neuronen interfereert met het onderzoek naar de vorming van synapsen, worden de cellichamen van neuronen direct naast elkaar geplaatst om een synaps te vormen. Op deze manier wordt neuronale uitgroei ontweken en kan dus de synaptogenese ongehinderd onderzocht kan worden. Het bestuderen van de excitatoire VD4-LPeD1 synaps heeft aangetoond dat de verantwoordelijke neurotransmitter acetylcholine is. Verder is gebleken dat de werking van trophe factoren noodzakelijk is voor het tot stand komen van deze synaps. Trophe factoren leiden tot de activatie van gentranscriptie en eiwittranslatie gemedieerd door activatie van Trk receptoren, zodat alle eiwitten die nodig zijn om excitatoire synapsen te vormen, geproduceerd worden.

Een andere karakteristieke eigenschap van evertebrate neuronen is dat axonen kunnen overleven zonder cellichaam. Geïsoleerde neuronen in kweek waarvan het cellichaam verwijderd en alleen het axon over is, kunnen overleven en vertonen zelfs uitgroei. Sterker nog, componenten van de eiwitsynthese machinerie zijn aanwezig in geïsoleerde axonen en het is bewezen dat er ook inderdaad eiwitten aangemaakt worden in axonale compartimenten. In dit proefschrift wordt de rol van locale eiwitsynthese, tezamen met de rol van trophe factoren in de ontwikkeling van synapsen nader bekeken. Daarnaast wordt ook de rol van trophe factoren in de overleving, uitgroei en het in stand houden van de neuronale integriteit van axonen zonder cellichaam onderzocht.

Samenvattend, in hoofdstuk twee wordt aangetoond dat bestaande synapsen een aantal dagen kunnen overleven zonder dat de cellichamen van de presynaptische en postsynaptische neuronen aanwezig zijn. Echter, indien de cellichamen niet aanwezig zijn, kunnen zich geen nieuwe synapsen vormen tussen axonen. Voor de vorming van nieuwe synapsen is het cellichaam van de presynaptische neuron een vereiste, terwijl het cellichaam van het postsynaptische neuron niet aanwezig hoeft te zijn. Het preparaat waar verder mee gewerkt is, bestaat uit een presynaptisch cellichaam en een postsynaptisch geïsoleerd axon. Uit hoofdstuk twee kan geconcludeerd worden dat geisoleerde axonen in staat zijn om bestaande processen zoals synaptische transmissie, te handhaven. Echter, voor de aanzet van nieuwe processen zoals de vorming van nieuwe synapsen, lijkt de aanwezigheid van zeker één cellichaam noodzakelijk te zijn.

In hoofdstuk drie wordt aangetoond dat uitsluitend in het presynaptische neuron gen transcriptie en eiwitsynthese noodzakelijk zijn voor de vorming van nieuwe synapsen, echter, in het postsynaptische axon dragen deze processen niet bij aan synaptogenese. Voorts wordt er ook in hoofdstuk drie aangetoond dat acetylcholine de neurotransmitter is die gebruikt wordt om signalen van de presynaptische cellichaam naar

het postsynaptische axon over te dragen. Deze resultaten zijn een sterke indicatie dat de synapsen die gevormd worden tussen het VD4 cellichaam en het LPeD1 axon gelijk zijn aan de synapsen die gevormd worden tussen de VD4 en LPeD1 cellichamen en aan de VD4-LPeD1 synapsen in het intacte brein.

In hoofdstuk vier wordt de rol van het axon uitgebreider onderzocht. Uit de vorige hoofdstukken is gebleken dat met name VD4 en trophe factoren een grote rol spelen in de vorming van synaptische connecties. Om de bijdrage van het axon aan de vorming van synapsen nader te bekijken, worden in hoofdstuk 4 de effecten van trophe factoren op het axon onderzocht. We hebben daarbij aangetoond dat trophe factoren alleen nodig zijn voor de uitgroei van axonen. Intrinsieke membraan eigenschappen zoals potentiaalrust membraan potentiaal, actiepotentiaal drempelwaarde en actie potentiaal amplitude zijn niet afhankelijk van trophe factoren gedurende de eerste 24 uur in kweek en in deze periode is de axonale synthese van eiwitten alleen van belang voor het onderhouden van de actiepotentiaal amplitude. Voorts is de expressie van de excitatoire acetylcholine receptoren niet alleen onafhankelijk van trophe factoren, maar ook van locale eiwitsynthese en Trk activatie. Dit is in tegenstelling tot het cellichaam van de LPeD1 neuronen, welke wel afhankelijk zijn van trophe factoren voor de expressie van excitatoire acetylcholine receptoren. Het verschil tussen cellichaam en axon blijft gehandhaafd in intacte LPeD1 neuronen (d.w.z. wanneer het cellichaam en axon in kweek niet van elkaar gescheiden worden), hetgeen aangeeft dat verschillende acetylcholine receptoren verspreid kunnen worden naar verschillende gedeeltes van het neuron. Samenvattend, voor de eerste 24 uur in kweek zijn trophe factoren nodig voor axonale uigroei, maar niet voor intrinsieke membraan eigenschappen en de expressie van excitatoire acetylcholine receptoren. Omdat trophe factoren wel nodig zijn voor de vorming van synapsen tussen de LPeD1 axon en VD4, lijkt het dat deze factoren van belang zijn voor andere processen in het axon, zoals het dirigeren van de receptoren naar de synaptische contactplaats met VD4.

In hoofdstuk 5 wordt aangetoond dat, naast acetylcholine receptor activatie, inderdaad trophe factoren en contact met specifiek de VD4 nodig zijn om axonale acetylcholine receptoren naar de synaptische contactplaats te herdistribueren. Verder wordt ook aangetoond dat axonen maar één synaps vormen, ook als er meerdere VD4s contact maken met het axon. Echter, intacte neuronen zijn in staat om meerdere synaptische connecties te vormen, zolang het axonale en somale compartiment van het intacte neuron maar met één enkele VD4 in contact is.

Samenvattend kan er geconcludeerd worden dat extrasomale compartimenten zonder cellichaam een zekere mate van onafhankelijkheid bezitten. Het onderhouden van bestaande processen kan door geïsoleerde axonen gedaan worden. Echter, nieuwe synapsvorming eist de aanwezigheid van zeker één cellichaam. Deze resultaten dragen bij aan de kennis in het functioneren van neuronen alsmede ook het autonoom functioneren van de afzonderlijke somale en axonale compartimenten. Deze kennis kan gebruikt worden om functioneren van zenuwcellen locaal te beïnvloeden of locaal te verbeteren in geval van ontregelde neuronale functie.

Abbreviations

ACh	acetylcholine
AChR	acetylcholine receptor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARIA	AChR-inducing activity
BDNF	brain-derived neurotrophic factor
CAM	cell adhesion molecule
ChAT	choline acetyltransferase
CM	conditioned medium
CNS	central nervous system
CPG	central pattern generator
DM	defined medium
ECM	extracellular matrix
EGF	epidermal growth factor
ELH	egg laying hormone
Eph receptor	erythropoietin-producing hepatocellular receptor
EPSP	excitatory postsynaptic potential
FM1-43	N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GlyR	glycine receptor
HB-GAM	heparin-binding growth-associated molecule
HMC	hexamethonium chloride
IEG	immediate early gene
IP3I	input 3 interneuron
Lav	lavendustin
L-EGF	*Lymnaea*-epidermal growth factor
LPeD1	left pedal dorsal 1
LTF	long-term facilitation
LTP	long-term potentiation
MASC	myotube-associated specificity component
Mec	mecamylamine
MEN1	menin 1
MuSK	muscle-specific kinase
Narp	neuronal activity-regulated pentraxin
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NMJ	neuromuscular junction
NT-3	neurotrophin-3
NT-4	neurotrophin-4
PSD-95	postsynaptic density 95
RMP	resting membrane potential
RPeD1	right pedal dorsal 1
RTK	receptor tyrosine kinase
SEM	standard error of the mean
Trk	tyrosine receptor kinase

VD4	visceral dorsal 4
VF	visceral F
YFP	yellow fluorescent protein

Dankwoord

Dankwoord.

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Ryanne